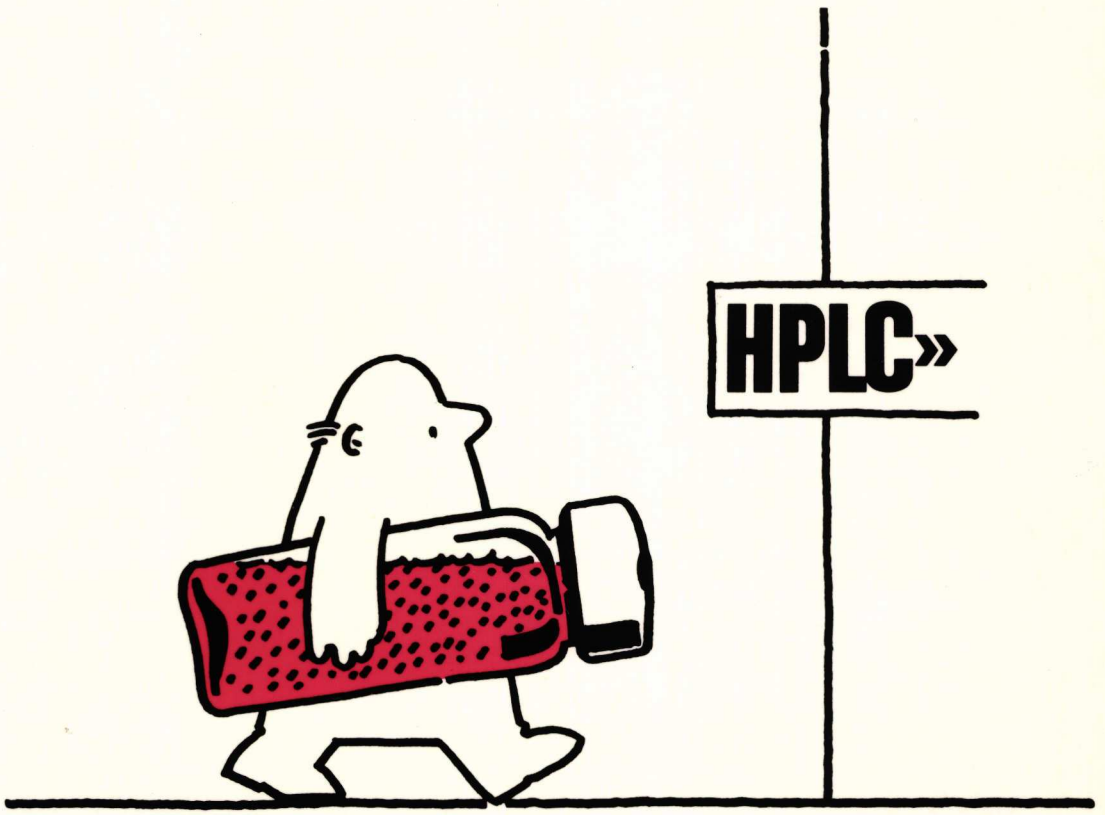
SELECTIVE ON-LINE PRECOLUMN SAMPLE HANDLING AND TRACE ENRICHMENT IN LIQUID CHROMATOGRAPHY



M.W.F. Nielen

Stellingen.

1. De tekst "...for ultraselective chemical isolation..." op een doos met reversed phase extractiekolommetjes is misleidend.

2. Er dient in het laboratorium meer aandacht besteed te worden aan de kwaliteit van het zogenaamde HPLC-grade water - met name bij het preconcentreren via solid phase extractie technieken.

3. Takeuchi et al.[1] hadden beter kennis moeten nemen van het werk van Van Vliet et al.[2], alvorens de bepaling van ftalaten in drinkwater te kiezen als toepassing voor on-line preconcentratie in microbore HPLC.
   (1) T. Takeuchi, Y. Jin and D. Ishii, J. Chromatogr., 321 (1985) 159.
   (2) H.P.M. van Vliet, Th.C. Bootsman, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 185 (1979) 483.

4. Het gebruik van korte GC-kolommen voor de analyse van weinig vluchtige organofosfor pesticiden wordt door Greve et al.[1] ten onrechte noodzakelijk geacht[2].
   (1) P.A. Greve and C.E. Goewie, Int. J. Environ. Anal. Chem., 20 (1985) 36.
   (2) H-J. Stan and D. Mrowetz, J. Chromatogr., 279 (1983) 173.

5. Bij het kiezen van I_{max}/k als criterium voor de geschiktheid van oxalaten voor HPLC/chemiluminescentie detectiesystemen, vergeten Honda et al.[1] het volume van de flow-cell[2].
   (1) K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177 (1985) 103.
   (2) G.J. de Jong, N. Lammers, F.J. Spruit, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 18 (1984) 129.

6. Het verdient de voorkeur in plaats van het begrip "detectiegrens" het begrip "bepalingsgrens" te gebruiken.

7. De frequentie van wetenschappelijke congressen dient primair bepaald te worden door de vorderingen op het betreffende vakgebied.

8. Gezien de mogelijke bijwerkingen en de stankoverlast, zou het gebruik van kreosoot

in hoestdranken verboden moeten worden.

9. Voor moderne analytische instrumenten geldt steeds vaker het gezegde van Tjeerd Zwaan: "As'r hier en deer moar 'n lampie brandt".

VRIJE UNIVERSITEIT TE AMSTERDAM

SELECTIVE ON-LINE PRECOLUMN SAMPLE HANDLING AND TRACE ENRICHMENT IN LIQUID CHROMATOGRAPHY

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De Boelelaan 1105

door

MICHAEL WILHELMUS FRANCISCUS NIELEN

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Promotor:	prof. dr. U.A.Th. Brinkman
Copromotor:	prof. dr. R.W. Frei
Referent:	dr. U.R. Tjaden

Aan mijn ouders

Contents.

Most of the contents of this thesis has been or will be published as regular contributions in scientific journals:

M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 317 (1984) 557. (section 2.1).
M.W.F. Nielen, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 57 (1985) 806. (section 2.2).
M.W.F. Nielen, J. de Jong, R.W. Frei and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 25 (1986) 37. (section 3.1).
J. de Jong, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 381 (1986) 431. (section 3.2).
M.W.F. Nielen, R. Bleeker, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 358 (1986) 393. (section 4.1).
M.W.F. Nielen, H.E. van Ingen, A.J. Valk, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr. (1986) in press. (section 4.2).
M.W.F. Nielen, R.E.J. van Soest, H.E. van Ingen, A. Farjam, R.W. Frei and U.A.Th. Brinkman (1986) in preparation. (section 4.3).
M.W.F. Nielen, E. Sol, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., 8 (1985) 1053. (section 5.1).
M.W.F. Nielen, R.C.A. Koordes, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 330 (1985) 113. (section 5.2).
M.W.F. Nielen, A.J. Valk, R.W. Frei and U.A.Th. Brinkman and Ph. Mussche, R. de Nijs and B. Ooms and W. Smink, J. Chromatogr, (1987) in press. (Chapter 6).

Chapter 1. General Introduction

1.1 Philosophy

Many sample preparation procedures include sample dissolution followed by liquid-liquid extraction. Traditional liquid-liquid extractions are performed in separatory funnels. Frequently, they are tedious, time-consuming and costly. These methods not only require several sample handling steps, but may present problems such as emulsifications, the evaporation of large solvent volumes, the disposal of toxic and inflammable solvents, impure and wet extracts, non-quantitative and irreproducible extraction yields. A highly promising approach is to enrich trace compounds of interest on suitable sorbents, in order to isolate and preconcentrate them prior to their separation and detection by means of a suitable chromatographic technique. Nowadays, this approach has become a popular topic in many reviews[1-6].

Sorbents such as carbon, alumina, silica, alkylsilane-modified silica or porous polymers are usually contained in a (plastic) cartridge or short stainless-steel or glass column, which are called solid phase extraction columns in general, or precolumns, when operated on-line with a chromatographic separation column. Depending on their characteristics (particle size of sorbent, length of sorbent bed, etc.), the column (or cartridge) can be operated at ambient pressure, i.e. under gravity-flow conditions, or at elevated pressure. Sample volumes are normally between about 1 ml and 1 l and the samples themselves are very diverse in nature: Aqueous samples (including surface waters and body fluids), organic extracts, gases, etc.

In this thesis, attention will be paid to the use of precolumns for the handling of organic trace compounds in environmental and biomedical, i.e., aqueous samples. Consequently, the solid phase extraction has been coupled on-line to a liquid chromatographic (LC) rather than a gas chromatographic (GC) system. Furthermore, the LC systems have been run in the reversed phase mode, i.e., where apolar-modified silicas are used as the stationary phase, and partly aqueous solvent mixtures as the mobile phase.

Functions which can be fulfilled by solid phase extraction columns are listed in Table I. Many papers on solid phase extraction techniques deal with off-line procedures. That is, the trace component is concentrated on a convenient and often large column and then eluted by a small volume of a suitable solvent. After further treatment - e.g., partial solvent evaporation or derivatization of the solute - an aliquot is injected into a chromatographic system. These off-line procedures have the inherent disadvantages of increase in method detection limit (injection of aliquot), losses due to thermal

decomposition (for thermolabile compounds, when an evaporation step is incorporated) and contamination risks, so the addition of an internal standard is often required. In addition, automation will be more difficult and demand the use of robotics[10].

By converting the off-line procedures into an on-line approach, many of the drawbacks can be avoided. The on-line approaches are called 'precolumn technology' which incorporates microprocessor-controlled switching of precolumn(s), samples, flush solvents, eluents, etc. Especially because of the automation aspect, precolumn technology allows the large-scale screening and monitoring of organic traces of interest. However, the chemistry and the principles of solid phase extraction are equal for both the off-line and the on-line procedures. Off-line methods may be easily converted into on-line procedures although the compatability of elution solvents with the mobile phase used for the analytical separation may cause problems.

Table I. Functions of solid phase extraction columns

Function	Comments	Ref.
Trace enrichment	Usually relatively apolar organic compounds from aqueous solutions, on hydrophobic sorbents. Enrichment factors of several orders of magnitude are easily obtained.	1-6
Clean-up	With hydrophobic sorbents only a partial clean-up will be obtained; consequently selective detectors and/or combinations with more selective sorbents are often required.	1-6
Storage of samples	Especially important when samples have to be taken at remote sites and long-term storage is unavoidable. Reduced volume and often improved stability of the samples.	7
Guard column	In on-line applications, precolumns which are eluted in the forward-flush mode (cf. section 1.4) act as a protection device for the much more expensive separation column.	
Derivatization	Sorbents with or without impregnated reagents act as a support for on-column derivatization.	8, 9

1.2 Principles of solid phase extraction

A column containing a suitable sorbent will trap the analyte (Fig. 1). The sample and solvents flow through the column by gravity or by positive-(syringe) or negative (vacuum manifold) pressure. In the case of on-line sample handling and trace enrichment, samples and flush solvents are applied via a second (low-cost) pump (Fig. 2, pump A). Elution can be done just by switching the valve to the 'inject' position. The elution can be performed either in the forward-flush, i.e., in the same direction as the sample loading, or in the backflush mode (opposite direction). The latter is realized by simply exchanging the connecting positions of pump B and the separation column at the switching valve (cf. Fig. 2).

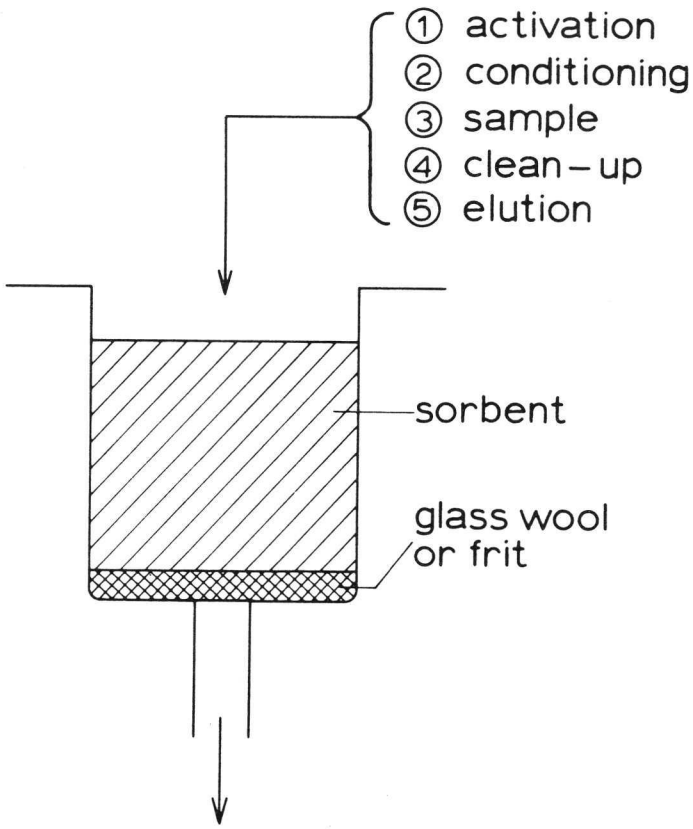
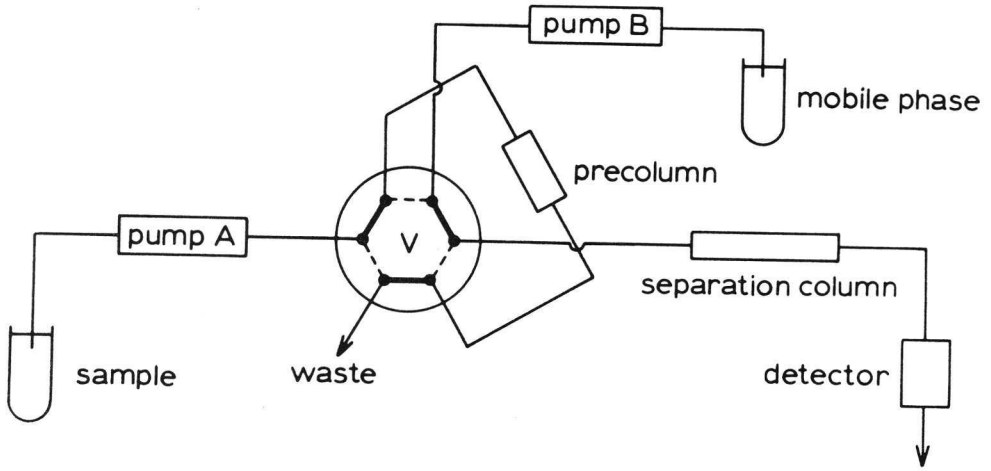


Fig. 1. Solid phase extraction sequence.

1) preconcentration step



2) separation step

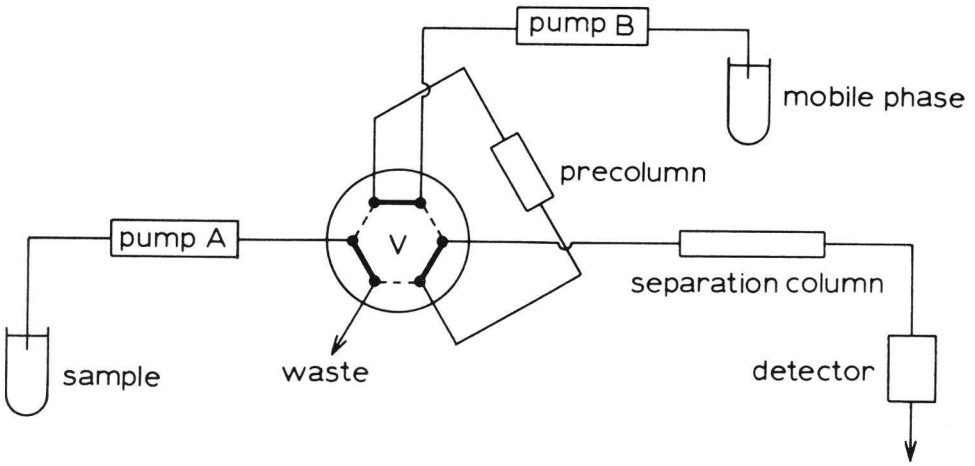


Fig. 2. On-line sample handling and trace enrichment using solid phase extraction. V = high-pressure switching valve.

A typical solid phase extraction sequence is as follows:

- Activation of the sorbent (wetting)
- Removal of the excess of activation solvent (conditioning)
- Sample application
- Removal of interferences (clean-up)
- Elution of concentrated analytes
- Regeneration of the column, if desired.

An example for a simple reversed-phase precolumn is given in Chapter 5. An octyl-bonded silica precolumn was wetted by the acetonitrile-containing mobile phase and conditioned with water. After the application of the plasma sample the precolumn was flushed with water to remove some interferences and finally the precolumn was eluted on-line by the mobile phase. Thereby the precolumn was wetted for the next extraction sequence.

When selecting a sorbent for solid phase extraction in a particular application, one has to take into account some physico-chemical considerations.

(a) The nature of the functional group(s) in the compound(s) of interest. The functional groups determine the affinity of a solute for a particular sorbent. Polarity and acidity of the functional group(s) have to be considered.

(b) The nature of the solvated (bonded) phase. The polarity, the possibility of ion-exchange in relation to the liquid (sample, flush solvent) phase determine the retention.

(c) The energetics of the interactions. Different interaction mechanisms with their corresponding energies are given in Table II. It can be seen that hydrophobic interaction (dispersion) by itself is a weak interaction and from the table one might conclude that covalent interaction should be preferred. However, one should realize that sample isolation by solid phase extraction is a combination of a retention and an elution process. In other words, covalent retention is attractive but efficient elution will be more difficult because of the energy required for braking the covalent bond. In practice, most applications deal with the sorption of organic compounds on hydrophobic surfaces or the formation of ionic bonds between ion-exchangers and acids or bases.

(d) The secondary interactions between the compound(s) of interest and the (bonded) phase. The retention of a solute can be based on more than one interaction. For example, with an ionogenic aromatic compound, the retention on a polystyrene-type ion-exchanger will primarily be governed by the ion-exchange process; π–π interaction will, however, also play a role. Another well-known example is represented by the presence of residual

silanol groups on chemically-bonded silicas and their affinity for, e.g., amines.

Table II. Energetics of interactions commonly employed in solid phase extraction

Interaction	Energy (kJ/Mole)
Dispersion	5-20
Dipole-induced dipole	8-25
Dipole-dipole	25-40
Hydrogen bond	25-40
Ionic	250-1050
Covalent	670-3360

(e) The interactions between the components of the sample matrix and the (bonded) phase. Unfortunately, solid phase extraction generally is not as selective as one would like it to be. In general, one should select the sorbent which shows sufficient retention abd selectivity for the analyte of interest and should not automatically select the sorbent which has the highest retention for the analyte, because it will often show high retention for many other interfering solutes as well.

(f) The interactions between the solute(s) and the sample matrix. Phenomena like drug-protein binding or adsorption of pollutants on sediments may influence the solid phase extraction efficiency. So in general, the recovery of known samples should be checked.

Considering the physico-chemical properties, one should optimize the solid phase extraction by carefully operating the available parameters.

Hydrophobic sorbents. Usually C_{18}-, C_8- or C_2-bonded silicas or styrene-divinylbenzene copolymers such as XAD-2 or PRP_1 are employed. Non-polar compounds and medium-polarity aromatics are effectively retained when water samples are applied to a hydrophobic extraction column. Partial clean-up may be obtained by flushing the loaded sample with water (including acids, bases, buffers, etc.), so long as the percentage of organic solvent in the water remains (very) low. Efficient elution may be performed (direct or stepwise) by an organic solvent such as hexane, chloroform, acetonitrile or methanol, or their mixtures. In the case of on-line precolumns, elution is almost invariably performed with the mobile phase used for the analytical separation,

usually mixtures of water and acetonitrile or methanol.

Ion-exchangers. Sulphonic acid or carboxylic acid functional groups, bound to silica or polymers, are used for strong and weak cationic extraction, respectively. Quaternary ammonium and tertiary or secondary amine bonded phases are used for strong and weak anionic extractions, respectively. Aqueous samples having a low ionic strength and a properly adjusted pH, are applied to the ion-exchanger. The loaded sample may be flushed with organic solvents or a (buffered) aqueous solution so long as the ionic strength remains low and the pH value remains in the proper range. Elution may be performed by a water or water/organic eluent with high ionic strength and/or a pH adjusted to neutralize either the analyte or the (weak) ion-exchanger.

Ligand-exchange. Davankov described ligand-exchange chromatography as a process in which interaction between the stationary phase and the solute of interest occurs during the formation of coordination bonds inside the coordination sphere of a complex-forming metal ion[11]. The metal ion is immobilized on a suitable support which may be either a silica- or polymer-based material having functional groups like ion-exchange sites or ligand-exchange sites. The metal ion itself should have enough coordination sites to be held by the support and to hold the analyte.

The aqueous or organic sample is applied to the extraction column from a solution which contains low concentrations of competing ligands, competing metal ions and inorganic ions which may strongly complex or form precipitates with the metal ion. In addition, the pH should be adjusted to prevent (hydr)oxide formation or loss of the metal from its support. The loaded sample may be flushed with organic and other solvents which meet the requirements of the sample solution. Elution can be done by changing the pH and/or introducing a stronger ligand (or an excess of a weak ligand) and/or introducing a solution of a competing metal salt.

Other solid phase extraction mechanisms fall outside the scope of this thesis. For more detailed information, the reader is referred to two excellent application books, which are available from manufacturers of solid phase extraction (off-line) columns[12,13]. In addition, one should consult a recent book about sample handling and selective detection in LC[14].

1.3 Breakthrough volume and capacity

At least two different processes have to be considered when one intends to prevent breakthrough of the analyte during the loading of a sample on the extraction column and during the flushing of the loaded sample in order to remove interferences, viz. the capacity and the retention.

(a) Capacity. The capacity of a solid phase extraction column depends on the type of stationary phase and the bed volume of the column. When manufacturer's data of the material are available, the capacity can often be simply calculated. Several columns packed with bonded silicas containing 200 mg of packing material were found to adsorb up to 3-12 mg of analyte without breakthrough[12]. Although we should consider the concentrations of both analyte and interferences, it is rather unlikely that in practical environmental, pharmaceutical and biomedical analyses - where concentrations typically are at the μg to ng per ml level - breakthrough will occur due to overloading of the column.

(b) Retention. Solid phase extraction is, in essence, ordinary liquid chromatography. Elution, expressed via the retention time or volume, is in principle independent of the solute concentration. This means that in solid phase extraction breakthrough can occur which is not due to overloading but to insufficient retention (i.e., too small a capacity factor, k') of the analyte in the phase system selected.

In reversed-phase liquid chromatography, retention data at a known percentage of organic modifier can, to a first approximation, be extrapolated to pure water or buffer solution, that is, to the conditions of sample application for reversed-phase extraction columns[15]. To give an example; if k' in, e.g., 50% modifier eluents already is in the k' > 5 range, then sufficient retention from water (0% modifier) may be expected on a much smaller (e.g., 50 times shorter) column of the same type because of the logaritmic relationship between k' and the modifier content.

However, reliable data on the retention of the solute of interest on a particular extraction column are not always available. For the accurate determination of the volume at which breakthrough occurs a so-called breakthrough curve should be recorded. This is illustrated for a simple (on-line) precolumn system in Fig. 3. After wetting and conditioning of the precolumn until baseline stability the solution of the solute of interest is fed straight to the detector to monitor the 100% breakthrough level. Then the valve is switched and the solute is adsorbed onto the precolumn; consequently, the detector response falls to a 'zero' level. At the point of breakthrough, the detector will start to produce a frontal analysis chromatogram which may be converted to an elution pattern by mathematical differentiation. The breakthrough volume can be read from the curve at the first deviation from the baseline level defined as, for example, the volume at which 1% breakthrough occurs.

If the breakthrough volume of a solute on a particular solid phase extraction column is known, one should choose the total sample plus flushing volume distinctly below the actual value of the breakthrough volume, as a safety margin against, e.g., packing

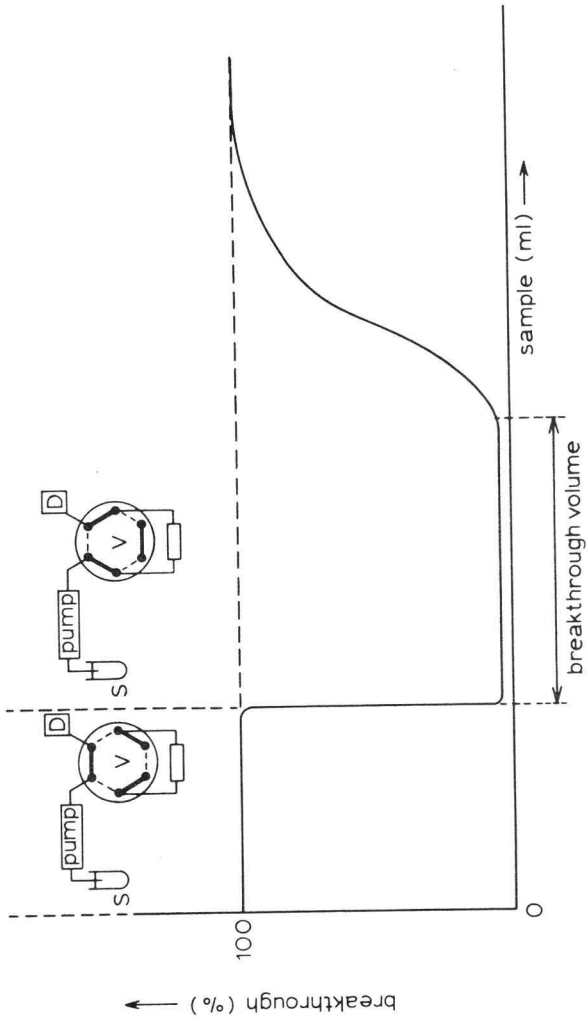


Fig. 3. Determination of the breakthrough volume via the recording of a breakthrough curve.

S = sample; V = high-pressure switching valve; D = detector.

irreproducibility and the presence of high concentrations of interfering contaminants.

As outlined above, it is common practice to pass as much sample volume (plus flushing volume!) through the solid phase extraction column as is possible, without allowing breakthrough and, thus, loss of recovery. Unfortunately for solutes with capacity factors of less than about 10 in pure water, the trace enrichment function is lost, since breakthrough occurs almost instantaneously. An alternative is to increase the size of the precolumn or, to overload the column so that the entire packing material is equilibrated with solute[16]. These methods show, however, some serious disadvantages. It is not possible anymore to flush the loaded sample to remove interferences, hence part of the clean-up function is lost. In addition, prior to elution, the column void volume is still filled with sample. When the (off-line) column is eluted with an organic solvent, the collected extract is therefore a mixture of, e.g., water-hexane, which hinders evaporation to dryness. Furthermore, with real samples it is important to assess the influence which other organic species in the matrix have on the capacity factor of the component of interest. Retention can be greatly diminished by high concentrations of other compounds and thereby provide irreproducible or very low enrichment factors. Finally, the absolute amount of solute trapped will be dependent on the amount of packing material in the solid phase extraction column making its production more critical.

1.4 Additional band broadening caused by on-line precolumns

With on-line solid phase extraction ('precolumn-technology') additional band broadening has to be considered. When the sample is transferred from the precolumn to the LC separation column by means of a suitable mobile phase, the separation should start with a narrow peak profile in order to keep total band broadening in the system as small as possible. In this respect, precolumn dimensions, particle size, particle porosity and the use of stationary phases in the precolumn and the separation column play an important role.

(a) Precolumn dimensions. The optimal volume of the precolumn is related to the volume and the plate number of the separation column and to the k' value of the analyte that is to be preconcentrated[17,18].
In general, the precolumn volume should be small as compared to the volume of the separation column and have a similar or smaller internal diameter. Longer precolumns should be used only in the backflush mode, i.e., when elution is done in the direction opposite towards the sample loading, but these columns are in general not recommendable[19].

When fast LC, microbore or highly efficient separation columns are being used, additional band broadening caused by the precolumn dimensions may become rather critical.

(b) Particle size. If the stationary phase in the precolumn is of the same type (and obtained from the same manufacturer!) as that in the separation column, then it is usual to use also the same (5-10 μm) particle size. However, other considerations such as low back-pressure and, thus, high sampling rates, manual packing and field sampling, and the prevention of clogging of the precolumn with dirty and viscous samples may force the analytical chemist to accept some additional band broadening for the benefit of larger particles (20-70 μm) in the precolumn[20,21].

(c) Combination of stationary phases. During the elution of the sample from the precolumn to the separation column, the capacity factor of the solute in the precolumn should be less than or equal to the k' in the separation column. Consequently, one should be aware of the additional band broadening which is introduced when not exactly the same stationary phase materials have been used in pre- and separation column. To quote an example, in general precolumns packed with styrene-divinylbenzene copolymers should not be combined with octadecyl-bonded silica separation columns because of the stronger affinity of the former towards non-polar and, especially, aromatic compounds. For the same reason, precolumns packed with strong ion-exchangers should not be combined with weak ion-exchange separation columns. But even combining two

octadecyl-bonded silicas obtained from different manufacturers may cause problems. Porosity, surface loading and end-capping may differ and cause severe additional band broadening.

1.5 Design of on-line precolumns

Precolumns for on-line solid phase extraction should have small dimensions to avoid additional band broadening during the elution towards the separation column. Furthermore it is recommended to use screens instead of frits to retain the packing material, in order to prevent blocking caused by, e.g., sample constituents. It should be easy to repack them and/or cartridges should be easily exchangeable manually or automatically.

Some commercially available precolumns for on-line sample handling and trace enrichment in liquid chromatography are listed in Table III. In this thesis, home-made precolumns[19] of 1-4.6 mm I.D. x 2-20 mm length were used which were hand-packed with either a thick slurry, by using a microspatula, or with a thin slurry contained in a disposable syringe. Alternatively, commercial Chrompack preconcentration columns were used.

1.6 Equipment for on-line solid phase extraction (precolumn technology)

Apart from the actual precolumn, one or more high-pressure switching valves, solvent selection valves, an additional pump and an auto-sampler may be necessary to use precolumn technology. The additional pump is especially important for carrying out precolumn conditioning, sample loading and flushing, during the separation of the previous sample on the main column. Usually, the equipment can be easily microprocessor controlled by an equivalent number of contact closures, which are nowadays standard on most integrators and computer systems. High-pressure switching valves and low-pressure solvent selection valves are commercially available from several manufacturers, for instance from Rheodyne (Berkeley, CA, U.S.A.). Alternatively, one can buy a column switching module. Several commercially available instruments are listed in Table IV. In practice we used either the Tracer MCS 670 or manually operated systems consisting of Rheodyne-type switching and solvent selection valves.

Table III. Precolumns for on-line sample handling and trace enrichment in liquid chromatography*

Name	I.D. (mm)	Length (mm)	Frit/Screen (F/S)	Prepacked: Silica, $C_{(1)8}$, SCX, SAX, Others					Hand-packing possible (yes/no)	Manufacturer
Uptight	2	20	F (2 µm)			—			Y	Upchurch Scientific Inc.
ManuFit MF II	4.6	5-20	S			—			Y	Bischoff GmbH
Brownlee	3.2	15	S	+	+	+	+	+	N	Brownlee Labs.
Guard-PAK	6	4	F	+	+			+	N	Waters Inc.
Chrompack pre-concentration column.	2	10	S	+	+	+	+		Y	Chrompack International

—, no data available
*The author and the Free University cannot accept any responsibility for data concerning commercially available equipment.

Table IV. Equipment for on-line sample handling and trace enrichment in liquid chromatography*.

Name	High-pressure switching valves	Low-pressure solvent selection valves	Additional pump included (Yes/No)	Microprocessor Int./Ext.	Manufacturer
Gynkotek	2	——	N	I	Gynkotek GmbH
WAVS	2	3 (2-port)	N	E	Waters Inc.
Tracer MCS 670	4	2 (6-port)	Y	E	Kontron Ltd.
MUST	2	opt. 1 (6-port)	N	E	Spark Holland B.V.
PROMIS	2	——	N	I	Spark Holland B.V.
PROSPEKT	3	3 (6-port)	Y	I	Spark Holland B.V.
HP	1	——	N	I	Hewlett Packard Inc.

——, not available.
*The author and the Free University cannot accept any responsibility for data concerning commercially available equipment.

1.7 Scope of this thesis and summary

When designing a liquid chromatographic system for automated organic trace analyses in aqueous samples one should develop a total-system strategy; that is, on-line sample handling, trace enrichment, analytical separation and final detection should not be considered as separate steps. The use of selective detection modes does not necessarily require selective sample handling techniques. Simple reversed-phase type precolumns will be sufficient in many cases. On the other hand however, when we intend to use simple detectors, e.g., UV absorbance, for trace analysis, care should be taken to obtain selectivity at the sample handling side by using selective sorbents and/or by coupling different types of precolumns in series.

Although some increase in selectivity may be obtained by replacing a C_{18} precolumn by a C_8-, C_4- or even C_2-bonded silica, this is not always possible because of the reduced breakthrough volume. In this work we applied ion-exchange and metal-loaded precolumns to obtain increased selectivity at trace levels.

Chapter 2 deals with cation-exchange precolumns. The requirement of low ionic

strength during the sample loading (cf. section 1.2) is fulfilled by introducing a precipitation step and complexation of interfering inorganic cations, prior to the actual on-line trace enrichment (section 2.1). Section 2.2 is an extension of the cation-exchange system towards on-line group separation of pollutants in industrial waste water by using precolumn technology with different types of precolumns in series.

Chapter 3 describes the selective on-line trace enrichment of phenol from river water (section 3.1) and the clean-up of barbiturates from urine (section 3.2) by anion-exchange precolumns. As an alternative to Chapter 2, interfering inorganic anions are removed on-line by trapping the analytes first on a hydrophobic precolumn, and performing a selective reconcentration on the ion-exchange precolumn.

Chapter 4 describes the use of metal-loaded precolumns for selective sample handling purposes. The basic applicability is demonstrated by a relatively simple application, viz. the on-line preconcentration of a thiol model compound on a mercury(II)-oxine phase (section 4.1). Section 4.2 deals with the evaluation of three different commercially available sorbents for ligand-exchange chromatography, applied to three different model systems, i.e., a thiol compound on mercury(II)-loaded precolumns, a compound having an ethynic bond on silver(I)-loaded and 4-chloroaniline on platinum(IV)-loaded precolumns. The environmental applicability of silver(I)-loaded precolumns is demonstrated for the phenylurea herbicide buturon. Section 4.3 describes a biomedical application. Synthetic steroids having an ethynic bond, are first enriched on a C_8 precolumn in order to remove interfering ligands with much higher complexation constants on the second precolumn, a silver(I)-oxine phase.

In Chapter 5 attention is paid to the miniaturization aspects of automated sample handling systems. Although liquid chromatographers are less optimistic about the microbore trend[22] as compared to a few years ago, there are still several application areas for miniaturized precolumn technology, for example in LC-MS, and LC-GC coupling. For environmental or biomedical trace analyses via microbore liquid chromatography (on-line) trace enrichment is even a must.

The selectivity in the microbore application (section 5.2) is simply obtained by applying a relatively large flush volume, after the loading of the plasma samples on a reversed phase micro-precolumn.

Chapter 6 describes a recent development in precolumn technology. It is not always possible or desired to re-use the precolumn (after on-line regeneration). We therefore developed an automated cartridge exchange system. Combined with a microprocessor controlled auto-sampler, this system can routinely handle plasma, serum and urine samples without requiring manual pretreatment steps.

Finally, in Chapter 7 some concluding remarks are made and the future of solid phase extraction and precolumn technology is briefly discussed.

1.8 References

1. M. Dressler, J. Chromatogr., 165 (1979) 167.
2. M.J. Fishman, D.E. Erdman and J.R. Garbarino, Anal. Chem., 55 (1983) 102R.
3. R.E. Majors, H.G. Barth and C.H. Lochmüller, Anal. Chem., 56 (1984) 300R.
4. J.R. Garbarino, T.R. Steinheimer and H.E. Taylor, Anal. Chem., 57 (1985) 46R.
5. R.W. Frei and U.A.Th. Brinkman, Trends in Anal. Chem., 1 (1981) 45.
6. R.W. Frei, M.W.F. Nielen and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 25 (1986) 3.
7. V.N. Mallet, G.L. Brun, R.N. McDonald and K. Berkane, J. Chromatogr., 160 (1978) 81.
8. M. Rosenfeld, M. Mareika-Russell and A. Phatak, J. Chromatogr., 283 (1984) 127.
9. A.H.M.T. Scholten, B.J. de Vos, J.F. Lawrence, U.A.Th. Brinkman and R.W. Frei, Anal. Lett., 13 (1980) 1235.
10. C.H. Lochmüller, K.R. Lung and M.R. Cushman, J. Chromatogr. Sci., 23 (1985) 429.
11. V.A. Davankov and A.V. Semechkin, J. Chromatogr., 141 (1977) 313.
12. Baker-10 SPE®, Applications Guide, Vol. I and II, J.T. Baker, Phillipsburg, NJ, U.S.A. (1984)
13. "Sorbent extraction technology", K.C. van Horne editor, Analytichem International, Inc., Harbor City, CA, U.S.A. (1985)
14. "Selective sample handling and detection in LC", K. Zech and R.W. Frei, editors, Elsevier, Amsterdam (in press).
15. C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 53 (1981) 2072.
16. R.E. Schoup and G.S. Mayer, Anal. Chem., 54 (1982) 1164.
17. L. Nondek and V. Chvalovsky, J. Chromatogr., 312 (1984) 303.
18. L. Nondek and V. Chvalovsky, J. Chromatogr., 268 (1983) 395.
19. C.E. Goewie, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 301 (1984) 325.
20. U. Juergens, Int. J. Environ. Anal. Chem., 25 (1986) 221.
21. K. Zech and R. Huber, J. Chromatogr., 353 (1986) 351.
22. J.H. Knox and K.W. Freebairn, presented at the Pittsburgh Conference (1985).

Chapter 2. Cation-exchange precolumns

2.1 *Use of cation-exchangers for the on-line preconcentration of polar anilines in liquid chromatography*

SUMMARY

On-line preconcentration (for use in liquid chromatography) of polar solutes such as substituted anilines cannot conveniently be carried out on conventional alkyl-modified silicas, or other, resin-based, hydrophobic materials because of insufficient retention. Small precolumns packed with a resin-based strongly acidic cation exchanger were successfully used for the preconcentration of nine polar anilines, with subsequent determination by liquid chromatography with UV absorbance or electrochemical detection.

Because of the low capacity of the ion exchanger and the presence of relatively high concentrations of ionic compounds in the surface water samples tested, it was necessary to introduce a clean-up step consisting of oxalate precipitation of calcium(II). With electrochemical detection, detection limits for the anilines are *ca.* 0.2–5.0 ng. In the analysis of river water samples, this corresponds to a detection limit of 0.02–0.5 ppb (10^9).

INTRODUCTION

Sample pretreatment based on liquid–solid sorption techniques has been shown[1] to be very useful for preconcentration of environmental samples in liquid chromatography (LC). In our laboratories, C_{18}-modified silica[2], styrene–divinylbenzene copolymers[3] and carbon-based[4] sorbents have been used for on-line trace enrichment of many non-polar and moderately polar solutes from aqueous samples, utilizing precolumns with geometrical volumes of 30–80 μl. Further work has shown, however, that such a set-up cannot be used for the efficient preconcentration of highly polar compounds from sufficiently large sample volumes. Such volumes, often arbitrarily set at *ca.* 10 ml, guarantee an enrichment factor of *ca.* 100 compared with a conventional 100-μl loop injection.

In the literature, precolumns packed with cation exchangers have been recommended for on-line trace enrichment of metal ions from nuclear reactor coolants and natural waters[5], and of amino acids and polyamines from blood and urine samples by means of an automated amino acid analyzer[6]. Sturgeon *et al.*[7] used columns containing silica-immobilized 8-hydroxyquinoline for the preconcentration of trace elements from sea water.

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Lores and co-workers[8,9] reported various LC procedures with electrochemical detection (ED) for the determination of halogenated anilines and related compounds with detection limits in urine of below 5 ppb*. A recent paper[10] describes the LC separation of phenylenediamines on a 15-cm column containing 3-μm C_{18}-modified silica, but no attempts to analyse real samples are presented. Kaczinsky *et al.*[11] used off-line preconcentration on a sulphonated XAD-4 resin, subsequent desorption with a mixture of methanol and ammonia, concentration via evaporation and final analysis by means of gas chromatography (GC). Over 50 organic bases were analysed with good recovery at the 1-ppm and 50-ppb levels, but no detection limits are given. Riggin *et al.*[12] evaluated the determination of aniline and its derivatives in waste water by means of GC and LC. However, aminophenols and phenylenediamines were not included in their study. The authors prefer GC with termionic nitrogen–phosphorus detection to LC with UV detection because of the distinctly higher sensitivity of the GC procedure (1–12 ppb). Finally, Riggin and Howard[13] developed a method for the determination of phenylenediamines in aqueous samples. Here, isolation on a strongly acidic cation exchanger preceded ion-pair LC with ED. However, no real preconcentration occurs in this procedure, and the limits of detection are still 10–60 ppb.

Obviously, there is still a need for an easily automatable LC method for the determination of polar anilines in real aqueous samples, *i.e.* in the presence of cationic and/or non-polar contaminants. In this paper, we describe such a procedure, which combines on-line preconcentration on a short precolumn, packed with a suitable cation exchanger, and separation on a C_{18}-bonded phase with ED.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system, consisting of two Model 410 pumps, a Model 200 programmer and MCS 670 Tracer switching unit, was used. In experiments aimed at obtaining maximum sensitivity, a laboratory-built syringe pump was used. A variable-wavelength LC 55 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) served as the UV detector. The electrochemical detector consisted of a Metrohm (Herisau, Switzerland) 1096/2 cell, equipped with a glassy carbon working electrode, a Ag/AgCl/1 *M* LiCl (in 50% methanol) reference electrode, and a copper or platinum auxiliary electrode, and a laboratory-built potentiostat/amplifier. Chromatograms were recorded on a W + W 900 (Kontron) recorder.

Stationary phases and columns

Preconcentration was accomplished on laboratory-packed[14] 2 × 4.6, 4 × 4.6 and 5 × 3.0 mm I.D. stainless-steel precolumns, which are also commercially available from Chrompack (Middelburg, the Netherlands). The precolumns were packed by using a microspatula, with the spherical 10-μm styrene–divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, U.S.A.) or a sulphonic acid-type silica-based (Merck SCX, 10 μm; Merck, Darmstadt, F.R.G.) or resin-based (Aminex A-7, 9 μm, Bio-Rad, Richmond, VA, U.S.A.) cation exchanger.

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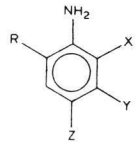
* Throughout this article, the American billion (10^9) is meant.

TABLE I

SUBSTITUTED ANILINES USED AS TEST COMPOUNDS



Compound	*X*	*Y*	*Z*	*R*	*Supplier*
Aniline	H	H	H	H	Aldrich
o-Phenylenediamine	NH_2	H	H	H	Fluka
m-Phenylenediamine	H	NH_2	H	H	Fluka
4-Methyl-*m*-phenylenediamine	H	NH_2	CH_3	H	Fluka
o-Toluidine	CH_3	H	H	H	Fluka
o-Anisidine	OCH_3	H	H	H	Fluka
p-Anisidine	H	H	OCH_3	H	Fluka
p-Chloroaniline	H	H	Cl	H	Fluka
p-Aminophenol	H	H	OH	H	Merck
3-Amino-4-ethoxyacetanilide	H	$NHCOCH_3$	H	OC_2H_5	Unknown

The analytical column was a 25 cm × 4.6 mm I.D. stainless-steel, or a 20 cm × 3.0 mm I.D. glass column, prepacked with 8-μm CP-Spher C18 (Chrompack).

Chemicals

Analytical-grade methanol, phosphoric acid, perchloric acid, potassium nitrate, potassium monohydrogen phosphate, citric acid and oxalic acid were obtained from J. T. Baker (Deventer, the Netherlands). EDTA was obtained from Sigma (St. Louis, MO, U.S.A.) and potassium citrate from ACF (Maarsen, the Netherlands). Demineralized water was purified in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system to obtain LC-grade water for use in mobile phases and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum.

The polar anilines used as test compounds, and their suppliers, are shown in Table I. Stock solutions were prepared by weighing the anilines and dissolving them in methanol. These solutions were diluted with LC-grade water adjusted to pH 3, to obtain standard solutions at the ppb level.

RESULTS AND DISCUSSION

Preconcentration

Breakthrough curves for PRP_1, Merck SCX and Aminex A7 columns were recorded according to the procedure outlined in ref. 2, with 250-ppb standard solutions of pH 3 at a flow-rate of 5 ml min^{-1}. The results are reported in Table II. Obviously, polar anilines cannot be concentrated from 10-ml sample volumes on the PRP_1 material. The only compound with appreciable retention is *p*-chloroaniline. Preconcentration of polar anilines on PRP_1 is more efficient under neutral conditions, as described elsewhere[15], but the breakthrough volumes are still rather low (0.3–24 ml). At pH 3, PRP_1 can be used as an effective clean-up filter, trapping non-polar

TABLE II

BREAKTHROUGH VOLUMES OF POLAR ANILINES ON SHORT PRECOLUMNS, PACKED WITH VARIOUS SORBENTS

LC-water samples, containing 250 ppb of test solute; pH adjusted to 3.0 with perchloric acid; sampling rate, 5 ml min^{-1}.

Compound	*Breakthrough volume (ml) on:*		
	PRP_1, 10 μm 2 × 4.6 mm I.D.	*Merck SCX, 10 μm* 4 × 4.6 mm I.D.*	*Aminex A-7, 9 μm* 4 × 4.6 mm I.D.*
Aniline	0	17	> 100
p-Phenylenediamine	0	> 100	> 100
m-Phenylenediamine	0	> 100	> 100
4-Methyl-*m*-phenylenediamine	1	> 100	> 100
o-Toluidine	1	65	> 100
o-Anisidine	0	34	> 100
p-Anisidine	0	34	> 100
p-Chloroaniline	6–10	–**	> 100
p-Aminophenol	0	9	> 100
3-Amino-4-ethoxyacetanilide	2	77	> 100

* Maximum values (see text).
** Not determined.

neutral contaminants without affecting the trace enrichment of the anilines on other column materials.

The silica-based Merck and, even more so, the resin-based Aminex cation-exchange columns, preconcentrate the protonated polar anilines well, with breakthrough volumes of more than 30 ml in all but two cases (Table II). Unfortunately, these breakthrough volumes have to be considered as maximum values. The depen-

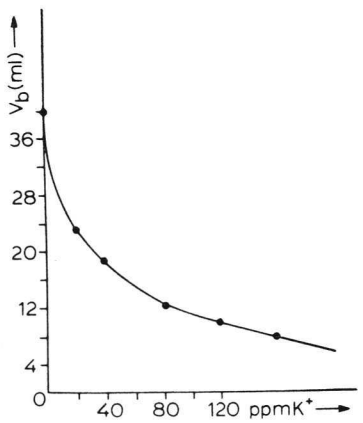


Fig. 1. Dependence of the breakthrough volume (V_b) of 3-amino-4-acetanilide on the ionic strength of the sample solution at pH 2.6. Solute concentration, 500 ppb; flow-rate, 2 ml min^{-1}; precolumn, 4 × 4.6 mm I.D., packed with Merck SCX.

dence of breakthrough volumes on the ionic strength of the sample solution was studied by recording breakthrough curves for 500-ppb solutions of 3-amino-4-ethoxyacetanilide at pH 2.6 to which increasing amounts of potassium nitrate had been added. The experiments were performed with the silica-based cation exchanger at a flow-rate of 2 ml min^{-1}. The breakthrough volume decreases dramatically with increasing ionic strength (Fig. 1). If a breakthrough volume of 10 ml is taken to be the lowest permissible value, then the highest allowable concentration of potassium is only 120 ppm. Bivalent ions, such as calcium(II), reduce the breakthrough volume to 10 ml at a concentration of 8 ppm. Passing several millilitres of a dilute solution of calcium(II) through a precolumn made on-line regeneration almost impossible: 20 ml of 10^{-3} *M* perchloric acid did not suffice to restore the original sorption quality of the cation exchanger. Finally, it should be noted that the breakthrough volume of 3-amino-4-ethoxyacetanilide in the absence of potassium nitrate decreases with decreasing pH. At pH 3.0 the breakthrough volume was 77 ml (Table II) and at pH 2.6 it was 40 ml (Fig. 1).

The low capacity of the silica-based cation exchanger prevents its use in small (4 × 4.6 mm I.D.) precolumns for the preconcentration of the anilines from real samples. The resin-based exchanger, which has a much higher capacity, was therefore used in all further work. A simple additional clean-up step was also introduced to reduce the negative influence of ionic sample constituents on breakthrough. It consists of precipitation of calcium(II) with oxalic acid and complexation of traces of iron(III) with EDTA. The procedure was developed with Amsterdam tapwater, which contains *ca* 100 ppm of calcium(II). Addition (per 100 ml of tap water) of 1.5 ml of a solution containing 2.4 mg of oxalic acid per ml and 1 ml of a solution containing 18 mg of EDTA per ml gave adequate results. To quote an example, after addition of the reagents to a spiked tap water sample, subsequent filtration through a 0.8-μm membrane filter, and adjustment of the solution to pH 3.0, the breakthrough of the spike, 4-methyl-*m*-phenylenediamine, on a 4 × 4.6 mm I.D. precolumn, packed with Aminex A-7, was more than 80 ml, which was the maximum volume tested. In another experiment, three consecutive 10-ml preconcentrations were carried out using the same spiked tap water; the precolumn was regenerated on-line with 20 ml of 0.02 *M* perchloric acid. Recoveries of over 90% were observed each time, indicating that the column had been successfully regenerated. The simple clean-up step does not impair the usefulness of the proposed method, since environmental samples are generally filtered anyway.

Desorption

For the desorption of aniline from the cation exchanger, 0.07 *M* aqueous potassium monohydrogen phosphate (adjusted to pH 7)–methanol (7:3) was used. For efficient desorption a fairly high potassium concentration of 0.1 *M* was found to be necessary. With a 0.1 *M* sodium (instead of a potassium) phosphate buffer the peaks were relatively broad and they tailed severely.

To prevent band broadening, the retention in the precolumn should be equal to or less than that in the analytical column. Unfortunately, the resin-based cation exchanger displays a stronger reversed-phase interaction with the anilines than does the C_{18}-bonded phase in the separation column. The resin-based exchanger will therefore cause more band broadening than the silica-based cation exchanger. How-

ever, aspects such as precolumn capacity and ease of column regeneration (see above) still make Aminex A-7 preferable to the silica-based exchanger. Backflush rather than forward flush desorption should be used because backflush seems to cause less broadening. The final gain in sensitivity was determined by comparing the peak heights obtained with 100-μl loop injections of a 1000-ppb solution of 4-methyl-*m*-phenylenediamine with those of the same solute enriched from 10 ml of a 10-ppb solution. Because of the additional band broadening (see above), the sensitivity was increased by preconcentration only 50- instead of 100-fold.

Final procedure

The automated analysis of the polar anilines was carried out with the experimental set-up shown in Fig. 2. The following procedure was adopted. (1) Precipitation and complexation of interferences by oxalate and EDTA, respectively. (2) Filtration and adjustment to pH 3.0. (3) Simultaneous clean-up and concentration on PRP_1 and Aminex A-7, respectively. (4) Flushing of the cation exchanger with water. (5) Backflush desorption from the cation exchanger to the C_{18} analytical column. (6) Flushing of PRP_1 with methanol. (7) Regeneration of cation exchanger with 0.02 *M* perchloric acid. (8) Regeneration of PRP_1 and flushing of cation exchanger with 10^{-3} *M* perchloric acid. (Note that steps 3–8 are fully automated; the time-based column switching programme is given in the Appendix.)

The PRP_1 precolumn upstream of the cation-exchange precolumn was included to act as a filter for non-polar and moderately polar compounds. In the absence of the PRP_1 precolumn, such compounds will be trapped by the resin matrix of the

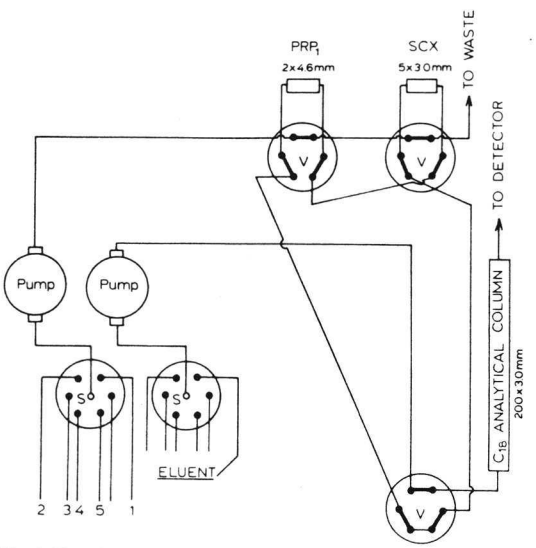


Fig. 2. Experimental set-up for the on-line preconcentration of water samples according to the final procedure described in the text. S = Low-pressure selector valve; V = high-pressure switching valve; 1 = sample; 2 = water; 3 = methanol; 4 = 0.02 *M* $HClO_4$; 5 = 0.001 *M* $HClO_4$.

cation exchanger and reduce the available ion-exchange capacity. As has been explained before, the protonated anilines are not significantly retarded on PRP_1 under the selected experimental conditions (Table II).

When large series of filtered tap or river water samples were handled, a gradual increase of the pressure over the analytical column was observed. This may have been caused by the presence of cationic sample constituents which precipitate as their phosphate salts. We therefore replaced the potassium phosphate by 0.07 *M* potassium citrate, adjusted to pH 6, which was used without any problem in all further work.

In the final procedure, a highly efficient 20 cm × 3.0 mm I.D. C_{18}-type separation column was used (at a flow-rate of 0.4 ml min^{-1}) instead of the earlier 25 cm × 4.6 mm I.D. column. This led to a two-fold increase in sensitivity, and to reduction of more than 50% in solvent consumption. It was found to be essential to match the inner diameter of the cation-exchange precolumn to the separation column (3 mm); otherwise very bad peak shapes will be obtained. Presumably, the highly different retention characteristics of the stationary phases in the precolumn and the analytical column play an important role here.

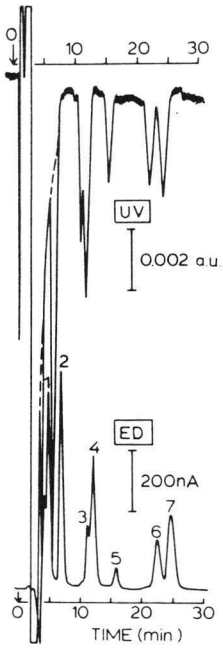


Fig. 3. LC of a standard solution containing 10 ppb of each of the compounds in Table I, except for the late-eluted *p*-chloroaniline; 10 ml of sample concentrated on a 5 × 3.0 mm I.D. Aminex A-7 precolumn. Analytical column: 20 cm × 3.0 mm I.D. CP-Spher C_{18}. Eluent: 0.07 *M* potassium citrate (pH 6)–methanol (7:3) at 0.4 ml min^{-1}. Detection by UV absorbance at 235 nm (attenuation, 0.02 a.u.f.s.) and, in series, electrochemically at +0.9 V (attenuation, 2 μA f.s.). 1 = *p*-Aminophenol + *m*-phenylenediamine; 2 = *o*-phenylenediamine + 4-methyl-*m*-phenylene-diamine; 3 = *p*-anisidine; 4 = aniline; 5 = 3-amino-4-ethoxyacetanilide; 6 = *o*-anisidine; 7 = *o*-toluidine.

Detection

Fig. 3 shows chromatograms of a standard solution containing 10 ppb of all test compounds except for the late-eluted *p*-chloroaniline. Two detectors in series, a UV-absorbance detector operated at 235 nm and an electrochemical detector operated at +0.9 V were used. The latter detector has higher sensitivity for the anilines (*cf.*, ref. 13) and better selectivity for the early-eluted test solutes. At the optimum oxidation potential, of +0.9 V, determined in preliminary experiments, the background current is low and the baseline therefore highly stable, which improves the signal-to-noise ratio of the anilines.

Repeatability ranged between ±1 and ±9% relative standard deviation (n = 4) at the 10-ppb level and was the same for loop injections and preconcentration experiments; this shows the excellent performance of the precolumn system. Detection limits with the electrochemical detector were between 0.2 and 5.0 ng for all test compounds. Method detection limits are summarized in Table III. The calibration curve for aniline was linear (r = 0.9989; six data points) over the whole range tested (0.5–50 ppb).

Application

Fig. 4 shows chromatograms of a blank Amstel (Amsterdam, The Netherlands) river water sample and the same sample spiked to 0.5 ppb with all but one of the test compounds. The early-eluted compounds are not easily detected because of the strong background in this region. The non-spiked river water contains *ca.* 0.5 ppb of aniline, *p*-anisidine and *o*-toluidine. Rather surprisingly, the phenylenediamines are hardly visible in the spiked sample. This is not due to the oxalate precipitation step or to coprecipitation, as was demonstrated in analyses of tap water samples with which no such complications occurred.

TABLE III

DETECTION LIMITS FOR ELECTROCHEMICAL DETECTION OF POLAR ANILINES IN THE PRECONCENTRATION/SEPARATION PROCEDURE

For conditions, see Fig. 4 and text.

Compound	*ED limit (ppb)**	*UV detection limit (ppb)**
Aniline	0.03	1.0
o-Phenylenediamine 4-Methyl-*m*-phenylenediamine	0.02	0.5
m-Phenylenediamine *p*-Aminophenol	0.04	–**
o-Toluidine	0.07	2.0
o-Anisidine	0.10	2.5
p-Anisidine	0.06	1.5
3-Amino-4-ethoxyacetanilide	0.50	2.5

* Signal-to-noise ratio, 3:1.

** Not determined.

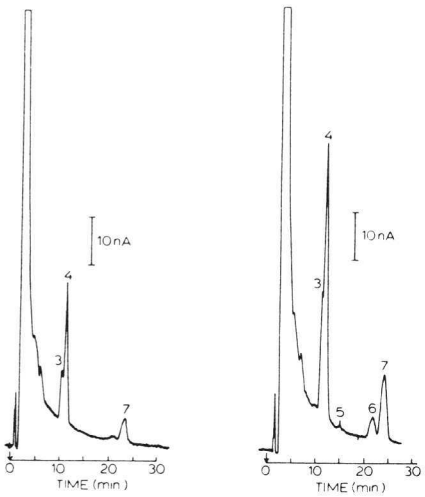


Fig. 4. Left: LC of a blank Amstel river water sample. Right: LC of an Amstel river water sample spiked with 0.5 ppb of each of the compounds in Table I except *p*-chloroaniline. Eluent pump, non-pulsating laboratory-built syringe pump. ED at +0.9 V (attenuation, 100 nA f.s.). Other conditions and identification as in Fig. 3.

CONCLUSIONS

Polar anilines can be preconcentrated on-line on a strongly acidic cation exchanger, provided a rapid clean-up step (oxalate precipitation and PRP_1 precolumn) is used to remove most of the interferences. The method can easily be automated and has sub-ppb detection limits for real samples when LC with ED is used.

Now that the preconcentration and on-line determination of these analytically problematic aromatic anilines have successfully been performed, an interesting next development will be to combine this knowledge with our know-how about on-line preconcentration of relatively non-polar compounds on PRP_1 and C_{18}-bonded phases. This should enable the development of an automated system for the on-line determination of a wide variety of cationic, neutral and anionic pollutants. The set-up of such a system and its application to the analysis of industrial waste water is presently being studied.

ACKNOWLEDGEMENT

Kontron is acknowledged for the loan of LC equipment and Hamilton, Merck and Chrompack for gifts of stationary phase materials and analytical columns.

APPENDIX

Automated procedure. Equipment: Kontron Model 200 programmer, Kon-

tron MCS 670 Tracer switching unit; analytical column, 20 cm × 3.0 mm I.D. CP-Spher C_{18}. Eluent, 0.07 *M* potassium citrate (pH 6.0)–methanol (7:3); flow-rate, 0.4 ml min^{-1}. Precolumn: 2 × 4.6 mm I.D. (PRP_1) and 5 × 3.0 mm I.D. (Aminex A-7); flow-rate as indicated below.

Time (min)	*Flow-rate (ml min^{-1})*	*Call file No.*	*Event*
0.0	8.0	88	Reset; flush capillaries with sample
0.7	2.5		
0.8	2.5	83	Sample over PRP_1 (4 ml)
2.4	1.7		
2.5	1.7	84	Sample over PRP_1 and Aminex A-7 (10 ml preconcentration)
8.5	0.0	94	
8.6	0.0	93	
8.7	0.0	86	Switch to water
8.8	8.0		Flush capillaries with water
9.5	1.0		
9.6	1.0	84	Flush Aminex A-7 with 1 ml of water
10.6	0.0		
10.7	0.0	83	
10.8	0.0	85	Flush capillaries with 0.4 ml of eluent
11.8	0.0	94	Desorb Aminex A-7 with 2 ml of eluent
16.8	0.0	95	
16.9	0.0	93	
17.0	0.0	86	Switch to methanol
17.1	8.0		Flush capillaries with methanol
17.8	2.5		
17.9	2.5	83	Flush PRP_1 with 1.5 ml of methanol
18.5	0.0	93	
18.6	0.0	86	Switch to 0.02 *M* perchloric acid
18.7	8.0		Flush capillaries with perchloric acid
19.4	1.7		
19.5	1.7	84	Regenerate Aminex A-7 with 20 ml of perchloric acid
31.5	0.0	94	
31.6	0.0	86	Switch to 10^{-3} *M* perchloric acid
31.7	8.0		Flush capillaries with perchloric acid
32.4	2.5		
32.5	2.5	83	Regenerate PRP_1 with 5 ml of perchloric acid
34.5	1.7		
34.6	1.7	84	Regenerate PRP_1 and flush Aminex A-7 (5 ml)
37.6	0.0		
37.7		End	

REFERENCES

1 R. W. Frei and U. A. Th. Brinkman, *Trends Anal. Chem.*, 1 (1981) 45.
2 C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 53 (1981) 2072.
3 C. E. Werkhoven-Goewie, W. M. Boon, A. J. J. Praat, R. W. Frei, U. A. Th. Brinkman and C. Little, *Chromatographia*, 16 (1982) 53.
4 W. Golkiewicz, C. E. Werkhoven-Goewie, U. A. Th. Brinkman, R. W. Frei, H. Colin and G. Guiochon, *J. Chromatogr. Sci.*, 21 (1983) 27.
5 R. M. Cassidy and S. Elchuk, *J. Chromatogr. Sci.*, 18 (1980) 217.
6 H. Adler, M. Margoshes, L. R. Snyder and C. Spitzer, *J. Chromatogr.*, 143 (1977) 125.

7 R. E. Sturgeon, S. S. Berman, S. N. Willie and J. A. H. Desaulniers, *Anal. Chem.*, 53 (1981) 2337.
8 E. M. Lores, D. W. Bristol and R. F. Moseman, *J. Chromatogr. Sci.*, 16 (1978) 358.
9 E. M. Lores, F. C. Meekins and R. F. Moseman, *J. Chromatogr.*, 188 (1980) 412.
10 *Supelco Reporter*, Vol. II, No. 2, Supelco, Bellefonte, PA, March 1983.
11 J. R. Kaczvinsky, K. Saitoh and J. S. Fritz, *Anal. Chem.*, 55 (1983) 1210.
12 R. M. Riggin, T. F. Cole and S. Billets, *Anal. Chem.*, 55 (1983) 1862.
13 R. M. Riggin and C. C. Howard, *J. Liquid Chromatogr.*, 6 (1983) 1897.
14 C. E. Goewie, M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 301 (1984) 325.
15 B. Zygmunt, J. Visser, U. A. Th. Brinkman and R. W. Frei, *Int. J. Environ. Anal. Chem.*, 15 (1983) 263.

2.2 Industrial wastewater analysis by liquid chromatography with precolumn technology and diode-array detection

ABSTRACT

Small precolumns packed with C18, PRP_1, and cation-exchange materials have been used for the on-line group separation and trace enrichment of industrial wastewater samples. The sample is divided into three main groups: a fraction containing nonpolar compounds, a second fraction containing medium polarity compounds and certain polar substituted aromatics, and a third fraction containing polar anilines and other polar bases. Each precolumn fraction is subsequently chromatographed on a C18 analytical column with a methanol gradient and detected by a diode-array UV-Vis detector. Multisignal plots and three-dimensional spectrochromatograms are used to get information about the identity of the pollutants present. The completely automated system is optimized for 29 selected chemicals of particular interest. Some of these compounds were found in the wastewater samples tested and were quantitated.

INTRODUCTION

Industrial wastewater represents a very complex matrix, containing organic pollutants over a wide range of polarity. There are several considerations for analyzing such samples: to check efficiency of production processes, to control the functioning of a wastewater treatment plant via fingerprinting techniques, and to maintain the stringent governmental rules for the discharge of industrial effluents into surface water and the environmental contamination with hazardous materials.

The complexity of the sample demands high resolution of the chromatographic system including the necessary sample pretreatment. Sample handling of wastewater was frequently done by liquid-liquid extraction[1,2] but such procedures made automation of the analysis more difficult. Recently, an on-line extraction and evaporation procedure was described for liquid chromatography[3], but this system seems to be rather complex. Nowadays, cleanup and trace enrichment via precolumn techniques is widely used in water[4] and biological[5-7] analysis. Although many sample pretreatments are still done with off-line disposable columns, the on-line techniques will be favored because of the automation aspect.

We analyzed industrial effluents by liquid chromatography with an on-line precolumn for trace enrichment[8] but the system described could only deal with (a small group of) specific compounds. By combining different types of precolumns, one should be able to handle wastewater samples containing pollutants of very different polarities.

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Little et al.[9] described a microprocessor controlled valve-switching unit for automated sample cleanup and trace enrichment in HPLC. Such equipment is very suitable for automated group separation and on-line trace enrichment on different types of precolumns.

In wastewater analysis, more sophisticated detection principles such as diode-array UV-Vis detectors may be helpful to search for particular pollutants in the samples and give a first indication about the identity of the substances present. If necessary, such detection techniques can be optimized to yield high signal-to-noise ratios for some compounds of special interest and/or can give information about peak inhomogeneity[10,11].

In the present paper both principles, precolumn technology for on-line group separation and trace enrichment and diode-array detection for peak and pattern recognition have been combined in an automated LC system for the analysis of 29 selected compounds in industrial wastewater.

EXPERIMENTAL

Apparatus. A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps, equipped with a high-pressure dynamic mixer and a pulse dampener, a MCS 670 Tracer valve switching unit, and a Model 200 programmer was used (this setup is shown in Figure 1). A HP 1040A (Hewlett-Packard, Palo Alto, CA, U.S.A.) diode-array UV-Vis detector was used with a HP 85 microcomputer, a HP 9121 dual disk drive, and a HP 7225 B plotter. Chromatograms were also analog recorded on a W + W 900 (Kontron) recorder.

Reagents. HPLC-grade methanol, perchloric acid, potassium acetate, acetic acid, and sodium oxalate were obtained from J.T. Baker (Deventer, The Netherlands). EDTA was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). All reagents but methanol were of analytical grade. Demineralized water was purified in a Milli-Q (Millopore, Bedford, MA, U.S.A.) filtration system to obtain LC-grade water for use in mobile phases and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum.

Twenty-nine pollutants were selected to be analyzed in the wastewater samples (Table I). These solutes were of more than 90% purity and supplied by Fluka, Merck, and Aldrich.

Stationary phases and columns. Wastewater samples were preconcentrated on homemade 2 x 4.6 and 4 x 4.6 mm I.D. stainless steel precolumns[12], which are also commercially available from Chrompack (Middelburg, The Netherlands). The precolumns were hand-packed with a microspatula, with 10 µm silica-based RP18 (Merck, Darmstadt,.

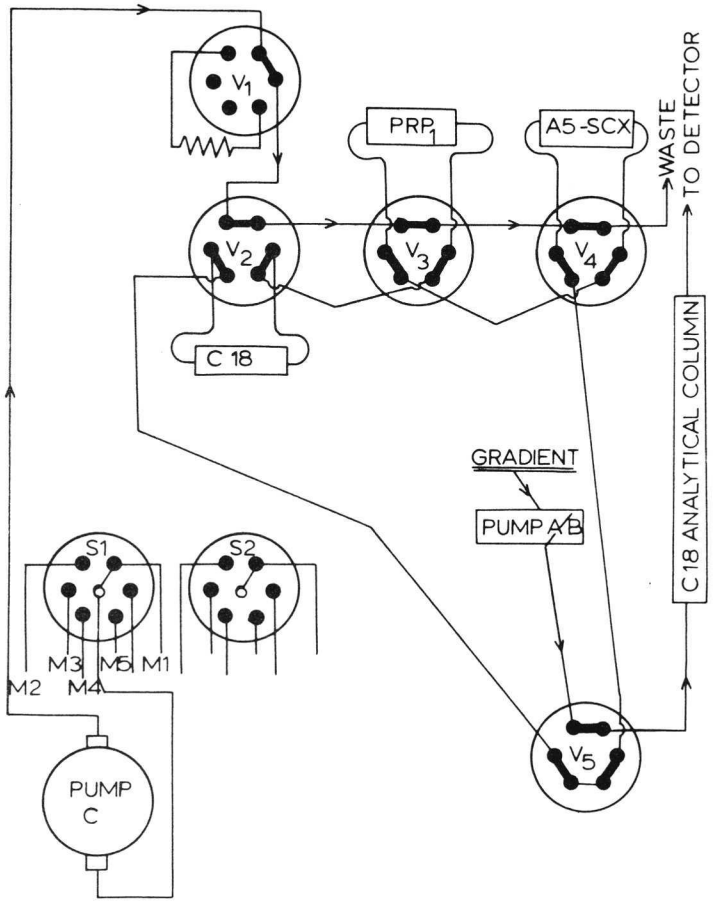

Fig. 1. Experimental setup for the on-line group separation and trace enrichment of wastewater samples: V, high pressure switching valve; S, low pressure selector valve; M1, sample; M2, 10^{-3} M $HClO_4$; M3, 50% methanol; M4, 0.02 M $HClO_4$.. Precolumns: 2 x 4.6 mm I.D. (RP 18), 4 x 4.6 mm I.D. (PRP_1), and 4 x 4.6 mm I.D. (Aminex A5). Analytical column: 25 cm x 4.6 mm I.D. CP-Spher C18.

F.G.R.), with the spherical 10 µm styrene-divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, U.S.A.), or with the 13 µm resin-based Aminex A5 (Bio-Rad, Richmond, CA, U.S.A.) sulphonic acid cation exchanger. The analytical column was a 25 cm x 4.6 mm I.D. stainless steel column prepacked with 8 µm CP-Spher C18 (Chrompack).

Procedures. Stock solutions of the selected pollutants were prepared by weighing and

Table I. Breakthrough volumes of 29 selected pollutants on short precolumns packed with various sorbents[a]

		breakthrough volume (mL) on		
no.	compound	RP 18, 10 µm 2 x 4.6 mm I.D.	PRP_1, 10 µm 4 x 4.6 mm I.D.	Aminex A5, 13 µm[b] 4 x 4.6 mm I.D.
1	p-aminophenol	0	0	> 100
2	p-phenylenediamine	0	0	> 100
3	m-phenylenediamine	0	1	> 100
4	4-methyl-m-phenylenediamine	0	1	> 100
5	o-phenylenediamine	0	1	> 100
6	aniline	0	2	> 100
7	p-anisidine	0	1	> 100
8	p-nitroaniline	1	10	> 100
9	3-amino-4-ethoxyacetanilide	1	7	> 100
10	o-anisidine	1	6	> 100
11	o-toluidine	1	3	> 100
12	picramic acid	2	> 100	-[c]
13	p-chloroaniline	2	30	-
14	p-nitrophenol	1	25	-
15	3,5-dinitro-o-cresol	10	> 100	-
16	m-cresol	1	37	-
17	nitrobenzene	2	> 100	-
18	p-chlorophenol	2	72	-
19	p-chloronitrobenzene	3	> 100	-
20	pentachlorophenol	> 100	-	-
21	o-dianidine	10	78	-
22	2-aminoanthraquinone	> 100	-	-
23	3,3'-dichlorobenzidine	72	-	-
24	3-amino-9-ethylcarbazole	50	-	-
25	p-aminoazobenzene	> 100	-	-
26	1-aminoanthraquinone	> 100	-	-
27	p-dichlorobenzene	17	-	-
28	2-phenylaminonaphtalene	> 100	-	-
29	1,2,5-trichlorobenzene	> 100	-	-

[a]LC-water samples containing 250 ppb of test solute; pH adjusted to 3.0 with perchloric acid; sampling rate 5 mL min^{-1}. [b]Maximum values (cf. ref. 13). [c]-, not determined.

dissolving them in methanol. These solutions were diluted with LC-grade water adjusted to pH 3.0 to obtain standard solutions at the parts-per-billion level. The wastewater samples were pretreated as follows: to 50 mL of (acidic) wastewater were added 5 mL of a saturated sodium oxalate solution, 0.75 mL of an EDTA solution (18 g/L), and 45 mL of a 10^{-3} M perchloric acid solution (see also Table II). The final solution was filtered over a 0.8 μm membrane filter to remove calcium oxalate (cf. ref. 13) and adjusted to pH 3.0 with perchloric acid, if necessary.

Table II. General procedure using the setup of Figure 1[a]

1	precipitation and complexation of interferences by oxalate and EDTA, respectively
2	filtration and adjustment to pH 3.0 (if necessary)
3	group separation and trace enrichment on C18, PRP_1, and Aminex A5 precolumns (in series)
4	flushing the C18, PRP_1, and Aminex A5 precolumns (in series) with 10^{-3} M perchloric acid
5	further cleanup of the cation exchanger by flushing with 50% methanol
6	backflush desorption from cation exchange fraction to C18 analytical column
7	backflush desorption from PRP_1 fraction to C18 analytical column
8	desorption from C18 precolumn to C18 analytical column
9	regeneration of the precolumns in series with 10^{-3} M perchloric acid

[a]Steps 3-9 are fully automated. The time-based column switching program is given in an appendix, which will be made available on request.

Breakthrough curves of the selected pollutants from the precolumns were recorded according to the procedure as outlined in ref. 14 with 250 ppb standard solutions (pH 3) at a flow rate of 5 mL min^{-1}.

The automated precolumn group separation and trace enrichment and the subsequent on-line gradient elution of the precolumns were performed by use of the experimental setup given in Figure 1. Five milliliters of sample was introduced via pump C to the three precolumns in series. The first, C18 precolumn trapped the nonpolar dye stuffs and other nonpolar solutes; the second, PRP_1 precolumn trapped the more polar aromatics like p-chloro- and p-nitrophenol and p-chloroaniline; the third, cation-exchange precolumn

trapped the polar phenylenediamines and other anilines. After this sample introduction the precolumns were flushed in series with 10 mL of 10^{-3} M perchloric acid to complete the desired group separation. Only the cation-exchange precolumn could be flushed with 3 mL of 50% methanol as an extra clean-up step.

Next, the ion-exchange precolumn was eluted on-line with the methanol-potassium acetate (pH 6) gradient to the C18 separation column. After the separation of this ion-exchange fraction, the PRP_1 and, finally, the C18 precolumn were eluted. The precolumns were regenerated on-line with dilute perchloric acid while analytical separations took place simultaneously. The entire procedure has been summarized in Table II.

Sample introduction, column switching, solvent selection, gradient elution, and start of detector and recorder were all controlled by the Model 200 Programmer. The diode-array detector was programmed to store four chromatograms (at 222, 390, 244, and 295 nm, respectively) and spectra (from 200 to 500 nm; at the peak apex and the base line after each peak) on flexible disk.

RESULTS AND DISCUSSION

Breakthrough volumes. Table I shows the retention behaviour of the 29 selected pollutants on the various precolumns connected in series (see also Figure 1) at pH 3. The samples are introduced through the precolumns in the order C18-PRP_1-Aminex A5. The nonpolar compounds (no. 20-29) are trapped on the C18 precolumn but the retention for p-dichlorobenzene and o-dianisidine is rather low. The o-dianisidine plus the chloro- and nitro-substituted aromatic compounds (no. 12-19) are, on the other hand, well retained on the PRP_1 material and can be preconcentrated on the PRP_1 precolumn. Of these, only 3,5-dinitro-o-cresol will also be partly retained on the C18 precolumn and hence be present in the C18 fraction. The remaining 11 compounds (1-11) which are polar anilines, can be successfully preconcentrated at pH 3 on the cation-exchange precolumn from relatively large sample volumes (cf. ref. 13). Of these, only nitroaniline will have been partly adsorbed on the PRP_1 precolumn.

To summarize, when the sample is pumped through the series of precolumns in the order C18-PRP_1-Aminex A5, a certain percentage of the compounds from the PRP_1 and Aminex A5 fraction will remain on the previous precolumns (C18 and PRP_1, respectively). When the precolumns are flushed in series with 10 mL of 10^{-3} M perchloric acid after the sample introduction, the desired group separation can be achieved. After this step, only p-nitroaniline and 3,5-dinitro-o-cresol appeared in more than one precolumn fraction.

Diode-array detection. Figure 2 shows a multisignal chromatogram obtained with 5 mL of standard solution (pH 3.0) containing 200 ppb of each of the 29 pollutants of interest. With the gradient profile, as included in this figure, acceptable resolution was obtained. The first 28 min of the chromatograms correspond to the ion-exchange precolumn (the polar aniline fraction). The period between 28 and 58 min corresponds to the PRP_1 precolumn (other medium polar aromatics) and the final period, from 58 to 90 min, to the C18 precolumn (nonpolar compounds). Trace B, the chromatogram recorded at 222 nm, shows all peaks of interest and can be regarded as a nonselective "total-peak chromatogram". It is clearly shown here that only p-nitroaniline (no. 8) and 3,5-dinitro-o-cresol (no. 15) appear in more than one fraction. Trace C at 390 nm gives a very selective chromatogram, only nitro aromatics and p-aminoazobenzene will appear. Traces D and E (at 244 and 295 nm) give additional information. The polar anilines (no. 1-11) for instance will appear at these wavelengths; their secondary maximum around 290 nm may be an aid for group identification. At 244 nm, p-chloroaniline (no. 13) will be relatively intense as compared to 222 nm which is verified by trace D. The dichloro- and trichlorobenzene (no. 27 and 29) show only a reasonable absorbance at low wavelengths; they appear only at 222 nm and not at one of the other wavelengths investigated.

These examples shows that multisignal plots obtained with diode-array detectors can be used to give preliminary information about the identity of the compounds present and such profiles may be helpful for peak recognition in unknown samples. Figures 3 and 4 represent spectrochromatograms of some parts of the same run. In the PRP_1 fraction (Figure 3) the nitro compounds (no. 8, 14 and 15) are recognized by their maxima at 400 nm. The picramic acid (no. 12) spectrum is clearly shown and a good example for the validity and usefulness of such plots for compounds with well-defined UV spectra. As a further example, compounds no. 24, 25, 27 and 28 can be identified with the aid of the 3D plot of Figure 4.

Application to real samples. Figure 5 is a multisignal plot obtained with a 5-mL industrial wastewater sample, collected after a biological step in the water treatment plant. The first part (the ion-exchange fraction) shows peaks at 222, 244, and 295 nm but not at 390 nm, indicating that there are no nitroanilines present but many other polar anilines. The spectrochromatogram of this region (Figure 6) shows the similarity in the UV spectra of these compounds, hence identification of these compounds is not possible via these spectra. On the other hand, comparison of retention times of polar anilines with the data obtained with well-defined standard samples will give more information. m-Phenylenediamine, aniline, and o-anisidine could be identified in this way. The second and third part of the chromatogram (PRP_1 and C18 fraction) contained only two peaks of

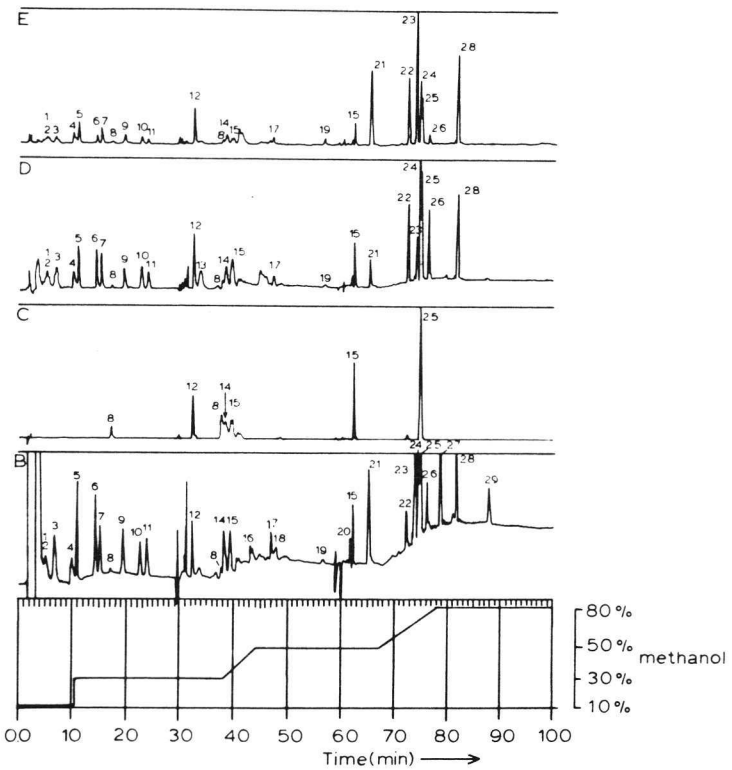

Fig. 2. Multisignal plot of a 5-ml standard solution containing 200 ppb of the selected pollutants from Table I. Gradient elution with 0.1 M potassium acetate (pH 6.0) and methanol (10-80%) as indicated. Detection at 222 (B), 390 (C), 244 (D), and 295 nm (E). Attenuation was 0.2 aufs. Peak numbers correspond to the compounds listed in Table I.

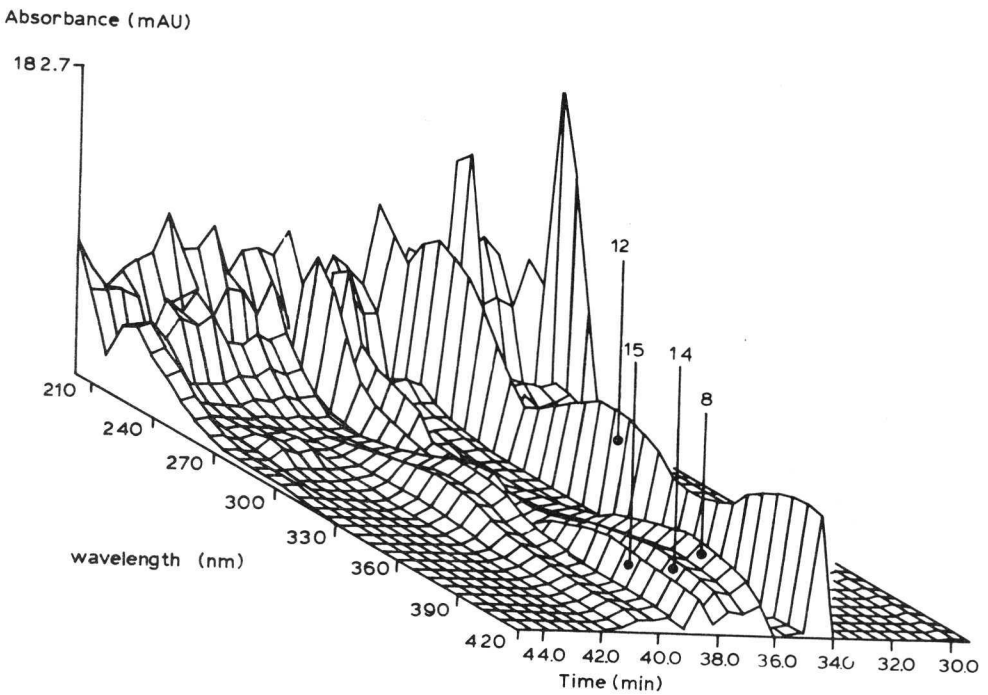

Fig. 3. Spectrochromatogram of a part of the PRP 1 fraction from the analysis of Figure 2. Spectra were memorized automatically at the peak apex and at the base line after the peak. Conditions are given in Figure 2.

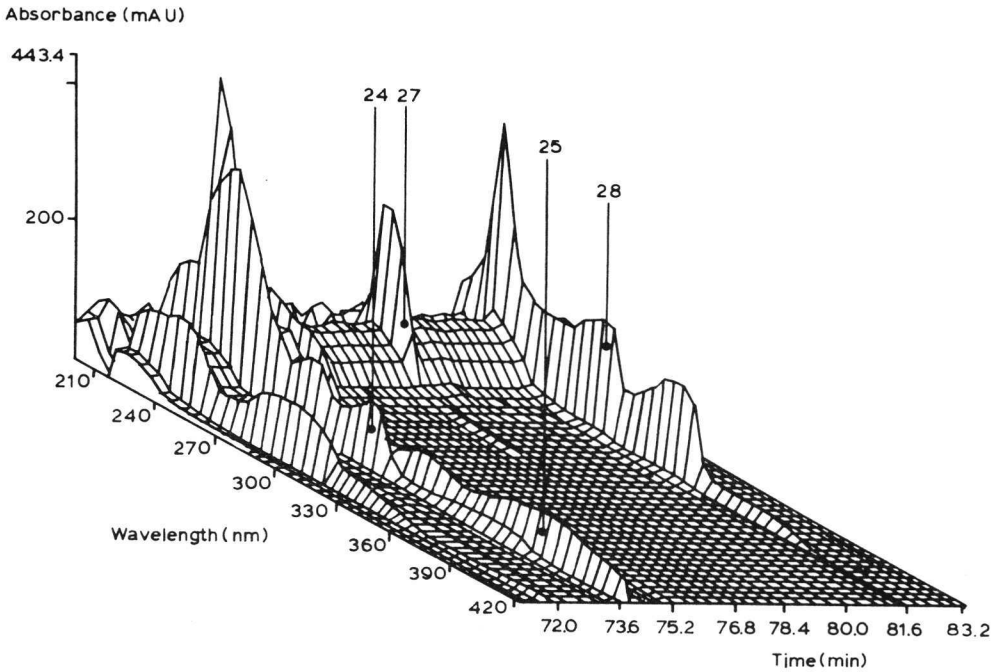

Fig. 4. Spectrochromatogram of a part of the C18 fraction from the analysis of Figure 2. Conditions are given in Figure 3.

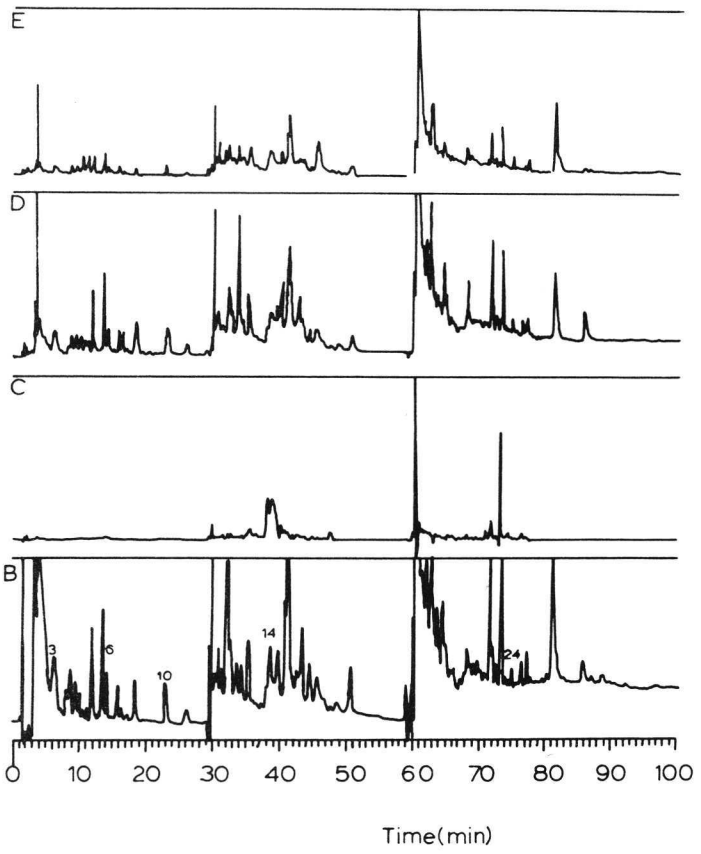

Fig. 5. Multisignal plot of a 5-ml industrial wastewater sample, taken after a biological treatment plant. Conditions are given in Figure 2.

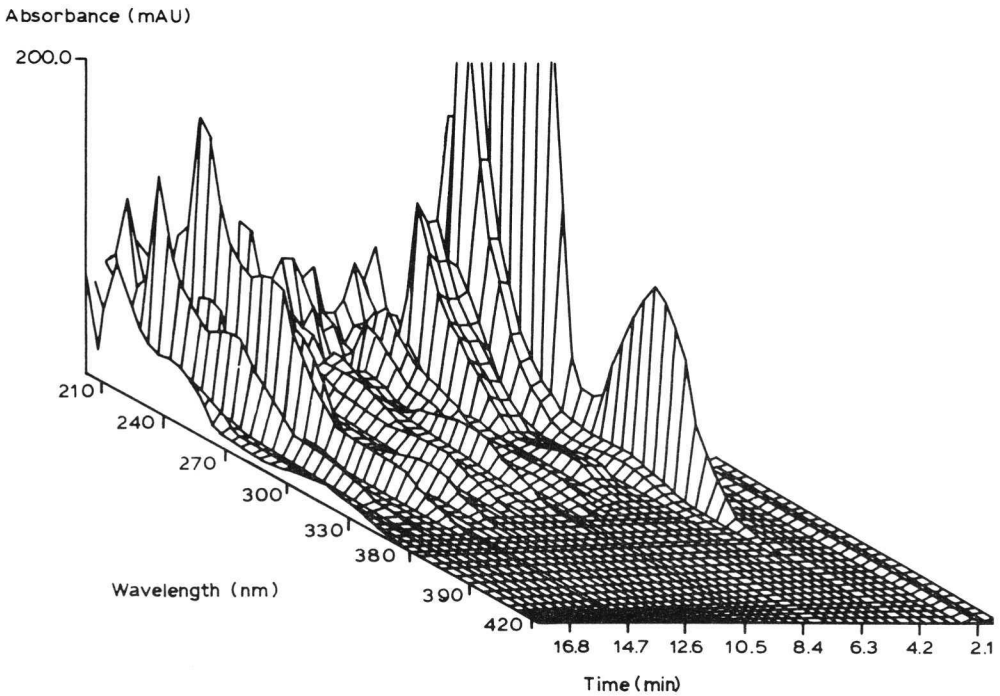

Fig. 6. Spectrochromatogram of a part of the Aminex A5 fraction from the analysis of Figure 5. Spectra were memorized automatically at the peak apex and at the base line after the peak. Conditions are given in Figure 2.

the pollutants of interest. p-Nitrophenol and 3-amino-9-ethylcarbazole were identified through their UV spectra. Spectra of the other peaks did not resemble the 29 reference spectra of the standard mixture, nor did their retention times.

Figure 7 was obtained by analyzing industrial wastewater before the treatment. It should be noticed that the chromatograms in Figure 7 are plotted at a two times reduced sensitivity of the detector, indicating that the UV absorbance of the pollutants is much higher before than after the biological treatment step, as was to be expected. Again, the aniline fraction was identified based on retention times and general spectral information. No p-nitroaniline was present but m-phenylenediamine, o-phenylenediamine, o-anisidine, and, especially, 4-methyl-m-phenylenediamine and aniline were present in relatively high concentrations. Unfortunately, there were no known species in the PRP_1 fraction, but in the C18 fraction o-dianisidine and p-dichlorobenzene could be identified.

The compounds tentatively identified in the wastewater sample which was collected after biological treatment (see Figure 5) were quantitated by standard addition: 100 ppb of the standard mixture was added to the wastewater sample which was reanalyzed. The concentrations were calculated from the relative peak heights with respect to the dilution factor and are given in Table III. The homogeneity of these spiked peaks was tested in the same experiment by memorizing also spectra on the slopes of the peaks and by plotting these spectra afterward overlapping each other. No peak inhomogeneity was observed in this experiment. Other peak homogeneity tests — such as ratio chromatograms — were obstructed by the background absorption of the mobile phase below 240 nm (caused by the high acetate concentration) which can lead to wrong conclusions about the peak homogeneity.

Table III. Concentration of identified pollutants in wastewater after biological treatment

compound	concn, ppb
m-phenylenediamine	262
aniline	150
o-anisidine	286
p-nitrophenol	260
3-amino-9-ethylcarbazole	58

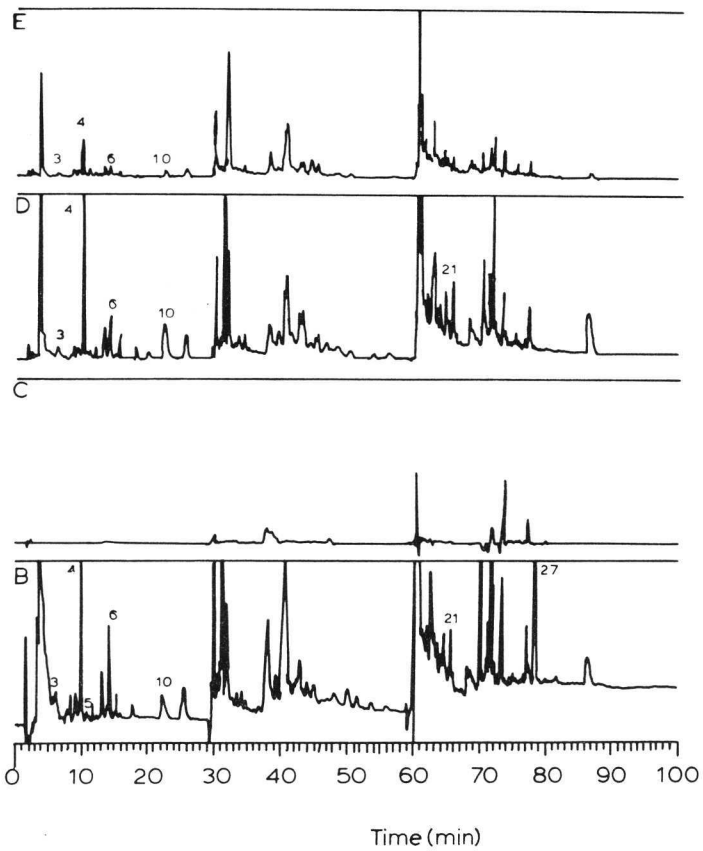

Fig. 7. Multisignal plot of a 5-ml industrial wastewater sample taken before a biological treatment plant. Attentuation was 0.4 aufs. Other conditions are given in Figure 2.

CONCLUSIONS

Precolumn technology offers the desired selectivity and sensitivity needed for industrial wastewater analysis with subsequent UV detection. Combination of different types of precolumns, optimized for pollutants of special interest, gives satisfactory group separations prior to the actual analysis. Unattended analysis is possible and easily done by using a time-based valve and solvent switching program.

The applicability of diode-array detectors to such complex matrices has been demonstrated. The choice of selective wavelengths and multisignal plotting may be an aid for a first identification. In addition three-dimensional spectrochromatograms will give UV spectra and the possibility of identification for those compounds having characteristic spectra. Of course systems like this can be made more powerful if more compounds present in wastewater are being considered. Spectra of unknowns can be compared automatically to reference data previously stored and peak inhomogeneity can be checked. Although UV spectra alone can never give absolute certainty about the identity of a compound, combination of selective sample handling with sophisticated detector software will reach the point where there is reasonable certainty about the compounds of interest. Further extension of this technique by including, i.e., functional group specific detectors such as electrochemical (cf. ref. 13) and reaction detectors, should provide additional evidence without necessarily having to resort to MS methodology.

ACKNOWLEDGEMENT

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References

1. R.M. Riggin, T.F. Cole and S. Billets, Anal. Chem., 55 (1983) 1862.
2. J. Folke, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 25.
3. K. Oka, K. Minagawa and S. Hara, Anal. Chem., 56 (1984) 24.
4. M.J. Fishman, D.E. Erdmann and J.R. Garbarino, Anal. Chem., 55 (1983) 102R.
5. C.E. Werkhoven-Goewie, U.A.Th. Brinkman, R.W. Frei, C. de Ruiter and J. de Vries, J. Chromatogr. Biomed. Appl., 276 (1983) 349.
6. B. Bjerg, O. Olsen, K.W. Rasmussen and H. Sorensen, J. Liq. Chromatogr., 7 (1984) 691.

7. J.W. Cox and R.H. Pullen, J. Chromatogr. Biomed. Appl., 307 (1984) 155.
8. B. Zygmunt, J. Visser, U.A.Th. Brinkman and R.W. Frei, Int. J. Environ. Anal. Chem., 15 (1983) 263.
9. C.J. Little, D.J. Tompkins, O. Stahel, R.W. Frei and C.E. Werkhoven-Goewie, J. Chromatogr., 264 (1983) 183.
10. B.J. Clark, A.F. Fell, H.P. Scott and D. Westerlund, J. Chromatogr., 286 (1984) 261.
11. A.F. Fell, H.P. Scott, R. Gill and A.C. Moffat, J. Chromatogr., 282 (1983) 123.
12. C.E. Goewie, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 301 (1984) 325.
13. M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 317 (1984) 551.
14. C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 53 (1981) 2072.

Chapter 3. Anion-exchange precolumns

3.1 Trace level determination of phenol by liquid chromatography with on-line precolumn technology and fluorescence detection

A strongly basic anion-exchange resin is used for the trace enrichment and automated sample handling of phenol, with subsequent determination by reversed-phase liquid chromatography with fluorescence detection. Because of the presence of high concentrations of ionic compounds in the water samples tested, phenol is first trapped on a relatively long precolumn filled with a highly hydrophobic packing material; during this step, (in) organic anions which are not retained, are flushed to waste. In the next step, phenol is desorbed from this column at high pH and sorbed in a small zone ("peak compression") on a short precolumn containing the anion exchanger.

In the analysis of tap and river water samples, the detection limit was found to be 10 ppt ($1:10^{11}$).

INTRODUCTION

Phenol and many of its chlorinated derivatives are present in (surface) water as a result of their frequent use in industrial activities and, in addition, because they are degradation products of several pesticides. In recent years, a number of chromatographic methods

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has been developed for the determination of chlorophenols in water samples. Buisson *et al.*[1] determined chlorinated phenols in aqueous samples by capillary gas chromatography with electron capture detection. Nair *et al.*[2] developed an isocratic liquid chromatographic (LC) system to separate 13 phenols on a reversed-phase column, and Shoup and Mayer[3] applied LC with electrochemical detection. Chriswell *et al.*[4] used an off-line preconcentration method using a $6 \times 1/2$ inch I.D. column packed with a resin-based anion exchanger. Phenols were sorbed at pH 12.5 and eluted with 4 M HCl, followed by liquid–liquid extraction, evaporation and, finally, gas chromatography. On-line trace enrichment and selective detection of chlorinated phenols was described by Werkhoven-Goewie *et al.*[5] who used a small precolumn packed with a styrene-divinylbenzene copolymer for preconcentration, a C18 separation column and a photochemical reaction detector in which the chlorinated phenols were dechlorinated on-line to obtain the highly fluorescent phenol. In this way (sub-) ppb levels could be selectively determined.

Many of the methods described so far do not include phenol itself and, if they do, the recovery is often low and/or the detection limit is rather high. In other words, there obviously still is a need for a—preferably automatable—LC method for the selective determination of sub-ppb levels of phenol in real samples, i.e., in the presence of ionic and non-polar contaminants. In this paper we describe a procedure in which phenol is, first, preconcentrated on a relatively long precolumn packed with a hydrophobic resin and—after the removal of inorganic compounds—transferred on-line to a second, small precolumn packed with a strongly basic anion exchanger. The LC separation is done on a C18 analytical column with subsequent fluorescence detection.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps, a pulse dampener, a MCS 670 Tracer valve switching unit and a Model 200 programmer was used; the set-up is shown in Figure 1. An LS-4 (Perkin-Elmer, Norwalk, CT, U.S.A.) fluorescence detector equipped with a 3 μl flow-cell was used for detection; the

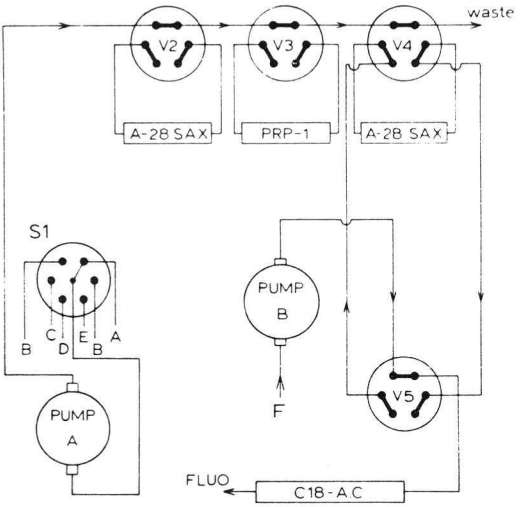

FIGURE 1 Experimental set-up for the on-line trace enrichment of phenol from water samples. S, low-pressure solvent selection valve; V, high-pressure switching valve. A, sample; B, water; C, sodium hydroxide (pH 11.5); D, 0.2 M sodium acetate containing 30% methanol; E, methanol–water (1:1); F, 0.4 M acetate buffer (pH 5.0)–methanol (1:1). Precolumns: 8 × 4.6 mm I.D. (Aminex A-28 at V_2), 20 × 4.6 mm I.D. (PRP_1 at V_3) and 10 × 3.0 mm I.D. (Aminex A-28 at V_4). Analytical column, 10 cm × 3.0 mm I.D. packed with CP-Spher C18.

excitation and emission wavelength were 271 and 297 nm, respectively. Chomatograms were analog recorded on a W+W 900 (Kontron) recorder and processed manually.

Stationary phases and columns

Trace enrichment was carried out on a 20 × 4.6 mm I.D. home-made stainless-steel precolumn,[6] packed with PRP_1 (cf. below), and on a slightly modified 10 × 3.0 mm I.D. Chrompack (Middelburg, the Netherlands) preconcentration column. The precolumns were hand-packed[6] by using a syringe filled with a slurry of either the spherical 10 μm styrene-divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, U.S.A.) in methanol or the 11 μm resin-based Aminex A-28

(Bio-Rad, Richmond, CA, U.S.A.) quaternary ammonium anion exchanger in an aqueous buffer.

The analytical column was a 100×3.0 mm I.D. glass cartridge prepacked with 8 μm CP-Spher C18 (Chrompack).

Chemicals

HPLC-grade methanol and water, analytical grade acetic acid, sodium acetate, sodium hydroxide, carbonate-free hydroxide "Dilut-it" and phenol were obtained from J. T. Baker (Deventer, the Netherlands). 4-Chlorophenol was obtained from Fluka (Buchs, Switzerland). Sodium hydroxide solutions were sealed with a calcium chloride cap to prevent carbon dioxide uptake by these solutions.

All eluents were degassed in an ultrasonic bath under vacuum.

Procedures

Stock solutions of phenol were prepared by weighing and dissolving in methanol, and stored at -20°C. The solutions were diluted with HPLC-grade water to obtain standard solutions at the (sub) ppb level. Breakthrough curves of phenol on the various precolumns were recorded according to the procedure reported in ref. 7, using 2–200 ppb standard solutions and a flow-rate of 2 ml min^{-1}.

Tapwater samples were analyzed without any pretreatment. The surface and waste water samples were filtered over a 0.8 μm membrane filter, in the latter case after 40–1,000-fold dilution with HPLC-grade water.

The automated sample handling and trace enrichment and the subsequent on-line elution of the sorbed phenol were performed using the experimental set-up given in Figure 1. Five ml samples are introduced via pump A onto the PRP_1 precolumn (at V_3, cf. Figure 1); phenol and many other organic compounds are retained but the inorganics, which would interfere in the trace-enrichment step on the anion-exchanger precolumn, are flushed to waste. Next, the PRP_1 precolumn is flushed with 3 ml of HPLC-grade water to ensure that all inorganics are flushed out. One now switches pump A to sodium hydroxide (pH 11.5), which, in order to remove systemic phenol impurities, first passes through an 8×4.6 mm I.D. anion-exchange precolumn (at V_2, cf. Figure 1) and then the PRP_1 precolumn.

Phenol is eluted from this PRP_1 precolumn in its anionic form and the phenolate ion is then sorbed on a small anion-exchanger precolumn (at V_4, cf. Figure 1) where peak compression occurs. Finally this precolumn is eluted via pump B, with a mobile phase consisting of 0.4 M acetate buffer (pH 5.0)–methanol (1:1), to the C18 separation column and phenol is detected in its neutral form.

The PRP_1 precolumn is regenerated during the analytical separation via pump A with methanol–water (1:1). The anion-exchange precolumn is regenerated by the mobile phase during the elution step. The other anion-exchange precolumn, used for reagent purification, is regenerated every five analyses via pump A with a 0.2 M sodium acetate solution (pH 7.0) containing 30% methanol. Finally the PRP_1 and both Aminex A-28 precolumns are flushed with water before the next sample is introduced. The entire procedure is summarized in Table I; details about the switching program can be found in the Appendix.

TABLE I

General procedure using the set-up of Figure 1.[a]

1. Trace enrichment of phenol on PRP_1
2. Flushing PRP_1 with water
3. Transfer of phenol to Aminex A-28 at pH 11.5
4. Flushing Aminex A-28 with sodium hydroxide (pH 11.5)
5. Forward-flush desorption from the anion-exchanger to the C18 analytical column
6. Regeneration of PRP_1 with methanol–water (1:1)
7. Flushing precolumns with water

[a]The time-based switching program is given in the Appendix.

RESULTS AND DISCUSSION

Retention of phenol on different precolumns

From earlier studies and preliminary experiments it is known that trace enrichment of phenol (in its neutral form) from 10 ml aqueous samples on styrene-divinylbenzene and other similar polymer sorbents is only possible when using relatively long precolumns of several centimeters. However, under these conditions many other organic compounds—both of a polar and non-polar nature—will

also be retained and, consequently, clean-up will be insufficient. Trace enrichment of phenol ($pK_a = 9.9$) in its anionic form can be carried out on an anion-exchange resin. However, the breakthrough volume will decrease rapidly if inorganic anions are present in the sample matrix and phenol dissolved in, e.g., saline is not retained at all.

By combining both types of packing materials mentioned above, one should be able to solve the various problems. As regards the trace enrichment of phenol on PRP_1, precolumn loading has to be done from an acidic or, preferably, a neutral solution. For example, on a 20×4.6 mm I.D. column, the breakthrough volume at pH 7 is already more than 20 ml. On the other hand, the breakthrough volume rapidly drops to about 1 ml or less for pH values higher than the pK_a of phenol. If phenol is eluted in its anionic form without using a modifier, i.e., by a steep pH increase only, many other organic compounds will remain on the polymeric resin. Phenol itself can be reconcentrated on a second anion-exchanger-containing precolumn from which it may be eluted in its neutral form onto a reversed-phase separation column. The neutral condition is optimal for the highly sensitive and selective fluorescence detection of the analyte. The proposed procedure effects an efficient removal of polar and non-polar (in)organic interferences; besides, the relatively broad phenol profile obtained on the PRP_1 precolumn will be compressed on the anion-exchanger in the second stage.

We preferred to use an aqueous alkaline solution of pH 11.5 instead of pH 12.0 for the transfer of phenol from the PRP_1 to the Aminex A-28 precolumn, becuase of the much lower breakthrough volume on the anion exchanger observed at pH 12.0 (6 vs. 20 ml), due to a stronger competition between hydroxyl and phenolate ions. Trace enrichment on a precolumn of the same dimensions (10×3.0 mm I.D.) but packed with a 40 μm silica-based instead of the 11 μm resin-based anion-exchanger was not successful: breakthrough occurred immediately.

Elution of phenol from the anion exchanger

Initially, desorption of phenol from the precolumn packed with Aminex A-28 was attempted with an aqueous solution containing 0.1 M sodium perchlorate and 0.01 M perchloric acid. This procedure

was not successful. Although the perchlorate acted as a displacer, phenol was found to be only partly converted into its neutral form by this solution, probably as a result of the large excess of hydroxyl ions released. In addition, regeneration of the precolumn became rather complicated. A mobile phase consisting of 1.0 M sodium acetate buffer (pH 5.0)–methanol (1:1) was found to provide an efficient elution of phenol in its neutral form. However, due to the high salt concentration, inner filter effects occurred during detection and the fluorescence yield was seriously decreased. Further work revealed that the best compromise between signal enhancement and efficiency of the elution procedure was obtained with a 0.4 M sodium acetate buffer of pH 5.0–methanol (1:1). In addition, with this mobile phase, regeneration of the precolumn was found to be superfluous.

Forward-flush elution was preferable to backflush elution, the latter showing a negative influence on band broadening due to the tendency to form a gap at the top of the precolumn. This is contrary to our earlier observations with cation-exchange resins.[8]

Purity of reagents

Sodium hydroxide pellets usually contain about 1% of sodium carbonate which, being a bivalent anion, will compete successfully with the phenolate anion. It can be calculated that 50 ml of 0.1 M sodium hydroxide (which was used in the preliminary experiments for precolumn regeneration) will be able to convert the anion-exchange precolumn completely into the carbonate form after which regeneration will be rather difficult. It is therefore essential to use carbonate-free sodium hydroxide solutions, which are commercially available under the tradename "Dilut-it". In addition, all solutions should be protected against atmospheric carbon dioxide as outlined in the experimental section.

Unfortunately, the carbonate-free sodium hydroxide solutions are sold in plastic containers, which were shown also to contain traces of phenol. This problem was solved by inserting a relatively large anion-exchange precolumn (8 × 4.6 mm I.D.) for on-line eluent purification during the phenol transfer (with sodium hydroxide pH 11.5) from the PRP_1 to the small anion-exchange precolumn.

General performance

Table II summarizes the analytical data obtained by the final procedure using the set-up of Figure 1 and the general procedure of Table I. It can be seen that traces of phenol can be successfully determined with good recovery. This is especially true when we keep in mind that phenol is preconcentrated and eluted twice and, in addition, the phenolate ions have to compete with hydroxyl ions which are present in 10^6-fold excess (as compared to 0.3 ppb anionic phenol) when phenol is reconcentrated on the anion-exchange precolumn!

TABLE II

Analytical data for the automated determination of phenol. Conditions: 5 ml sample solutions analyzed according to the procedure of Table I (cf. Figure 1); data based on peak area measurements

Criterion	Level	Result
Repeatability	0.3 ppb ($n=10$)	$\pm 2.4\%$
Recovery	0.4 ppb 4.0 ppb	89.5% 86.7%
Linearity (r)	0.03–100 ppb ($n=7$)	0.9994
Detection limit	S/N = 3/1	50 pg or 10 ppt

Application to real samples

Tap water (Free University) was analyzed and found to contain less than 0.01 ppb phenol. River water (River Waal, Lobith, the Netherlands) contained approximately 0.1 ppb phenol. Figure 2 shows chromatograms of river water, tap water and HPLC-grade water, respectively, spiked with 0.4 ppb of phenol. The selectivity of the method is clearly demonstrated at this trace level. The repeatability of the method in analyzing river water samples was found to be $\pm 4\%$ RSD ($n=5$) at the 0.5 ppb level. The recovery of spiked river water as compared to spiked HPLC-grade water was $101 \pm 4\%$ ($n=3$).

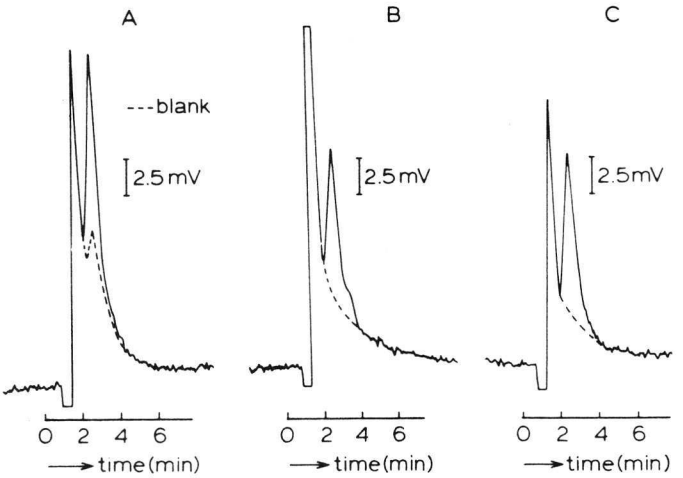

FIGURE 2 Chromatograms of 5 ml water samples without added phenol (-----) and spiked with 0.4 ppb phenol (———), analyzed using the set-up of Figure 1 and the general procedure of Table I. (A) Waal river water; (B) tap water; (C) HPLC-grade water standard solution; Analytical column, 10 cm × 3.0 mm I.D. CP-Spher C18. Eluent, 0.4 M acetate buffer (pH 5.0)–methanol (1:1) at 0.4 ml min^{-1}. Detection by fluorescence at 271 nm excitation and 297 nm emission wavelength. Other conditions as in Figure 1.

We also analyzed several industrial waste water samples for phenol and found concentrations of between 60 ppb and 6 ppm. Blank urine (after hydrolysis with sulphuric acid to release phenol from its conjugates) contained 1 ppm phenol, which is a well-known metabolite of tyrosine. However, one should realize that for such high levels other, less expensive methods, are available. The present method should be considered as a procedure for the selective determination of real trace levels in aqueous samples.

The excellent selectivity of the proposed method is demonstrated by comparing Figures 2 and 3. The latter one represents the preconcentration of phenol from 5 ml water samples from two different rivers on the non-selective PRP_1 precolumn with an additional flush step of 6 ml HPLC-grade water. The anion-exchange precolumns were not used in this case and the phenol was simply

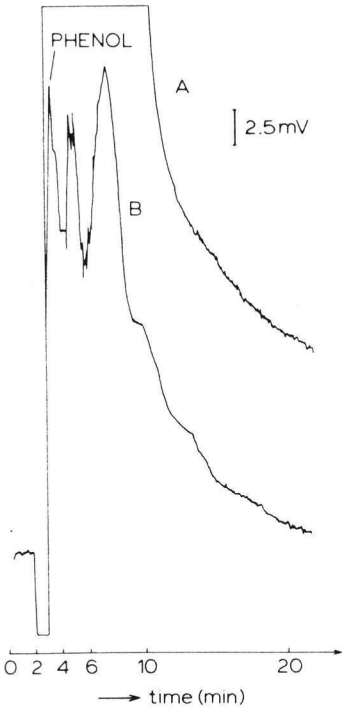

FIGURE 3 Chromatograms of 5 ml river water samples spiked with 0.4 ppb of phenol obtained after trace enrichment on a 20 × 4.6 mm precolumn packed with PRP_1, a flush with 6 ml of water and direct elution to the analytical column with methanol–water (1:1). Other conditions as in Figure 2. Samples taken from the rivers Amstel (A) and Waal (B).

eluted with 50% methanol onto the C18 separation column. We can see that the combined use of fluorescence detection and an anion-exchange precolumn combined with PRP_1 results in such an increase in selectivity, that trace-level determination of phenol in real samples is easily attainable.

To demonstrate the selectivity of the present method for phenol over other—e.g., lower chlorinated—phenols, we analyzed a sample

spiked with a 100-fold excess of 4-chlorophenol. No peak due to the latter compound was observed.

CONCLUSIONS

Trace levels of phenol in water samples can be selectively determined by liquid chromatography with on-line precolumn trace enrichment and clean-up, and fluorescence detection. The combination of a relatively large non-selective precolumn, packed with PRP_1, and a small precolumn packed with selective material such as an ion-exchanger, seems to be a generally applicable approach to deal with organic and inorganic interferences.

The described method is linear over almost four orders of magnitude and allows the detection of phenol in environmental samples at the parts-per-trillion level with a recovery of approximately 90%. The applicability to the selective sample handling of anionic drugs in biomedical samples with ordinary UV detection is presently being studied.

Acknowledgements

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References

1. R. S. K. Buisson, P. W. W. Kirk and J. N. Lester, *J. Chromatogr. Sci.* **22,** 339 (1984).
2. J. Nair, K. M. Munir and S. V. Bhide, *J. Liq. Chromatogr.* **6,** 2829 (1983).
3. R. E. Shoup and G. S. Mayer, *Anal. Chem.* **54,,** 1164 (1982).
4. C. D. Chriswell, R. C. Chang and J. S. Fritz, *Anal. Chem.* **47,** 1325 (1975).
5. C. E. Werkhoven-Goewie, W. M. Boon, A. J. J. Praat, R. W. Frei, U. A. Th. Brinkman and C. Little, *Chromatographia* **16,** 53 (1982).
6. C. E. Goewie, M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.* **301,** 325 (1984).
7. C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.* **53,** 2072 (1981).
8. M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.* **317,** 557 (1984).

Appendix

Automated procedure. Equipment: Kontron Model 200 programmer, Kontron MCS 670 Tracer switching unit; analytical column, 10 cm × 3.0 mm I.D. CP-Spher C18. Eluent, 0.4 M sodium acetate (pH 5.0) Methanol (1:1); flow-rate, 0.4 ml min^{-1}. Precolumns: 8 × 4.6 mm I.D. and 10 × 3.0 mm I.D. (Aminex A-28); 20 × 4.6 mm I.D. (PRP_1), flow-rate, Pump A, as indicated below.

Time (min)	Flow-rate (ml min^{-1})	Call file no.	Event
0.00	5.0		Flush capillaries with sample
1.95	2.0		
2.00	2.0	83	Sample over PRP_1 (5 ml)
4.50	2.0	93	Reset V_3
4.51	2.0	86	Switch to water
4.52	5.0		Flush capillaries with water
5.45	2.0		
5.50	2.0	83	Flush PRP_1 with 3 ml water[a]
7.00	2.0	93	Reset V_3
7.01	2.0	86	Switch to NaOH, pH 11.5
7.02	5.0		Flush capillaries with NaOH
7.95	1.5		
8.00	1.5	82	On-line purification of NaOH
8.50	1.5	84	Equilibrate A-28
8.51	1.5	85	Flush capillaries with eluent
8.95	1.2		
9.00	1.2	83	Transfer sample from PRP_1 to A-28 (3.6 ml)
12.00	1.2	93	Flush A-28 with 1.2 ml NaOH
13.00	1.2	94	Desorb A-28 with 3 ml eluent
13.10	1.2	92	All precolumns reset
13.20	1.2	86 dur 2	Switch to 50% methanol
13.30	5.0		Flush capillaries with 50% methanol
14.20	3.0		
14.30	3.0	83	Regenerate PRP_1 with 18 ml 50% methanol
17.50	3.0	86	Flush PRP_1 with 8 ml water
20.50	1.5	93	Reset V_3
20.60	1.5	84	Flush A-28 with 4.5 ml water
23.50	0.0		Stop pump A
23.60	0.0	88	Reset all
23.70	0.0	End	

[a]10 ml for river water samples.

3.2 *Selective on-line sample handling for the determination of barbiturates in urine by liquid chromatography with precolumn technology*

Despite their widespread replacement with benzodiazepines in the field of hypnotic formulations, barbiturates are still being prescribed extensively. Monitoring of barbiturate concentrations in body fluids is important because of their therapeutic width, which is quite small [1]. In addition, barbiturates are frequently used for suicidal attempts, hence barbiturate screening is quite common in forensic research.

Gas chromatographic (GC) and reversed-phase column liquid chromatographic (LC) methods for the determination of barbiturates were recently reviewed by Gupta [2] and by Heusler [3]. The use of LC in the determination of barbiturates in body fluids suffers from the major drawback that non-selective low-wavelength UV detection has to be used, which hampers on-line analysis of real samples. In principle, the selectivity can be somewhat improved by determining the barbiturates in their anionic form at 254 nm [4–6]. However, we preferred to incorporate the selectivity in the sample handling step by on-line solid-phase extraction of the barbiturate anions using an anion-exchange resin. Interfering inorganic ions are eliminated in the same way as has been demonstrated recently in the trace analysis of phenol in surface water [7], viz. via a solid-phase extraction procedure involving a hydrophobic sorbent.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps (one of them equipped with a home-made membrane pulse dampener),

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an MCS 670 tracer valve-switching unit and a Model 200 programmer, was used in combination with a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 UV detector operated at 220 nm. Chromatograms were analogue recorded using a Kipp & Zonen (Delft, The Netherlands) BD 8 recorder and processed manually.

Stationary phases and columns

Sample clean-up was performed on home-made 2 × 4.6 mm I.D. and 5 × 3.0 mm I.D. stainless-steel precolumns [8] packed by hand, using a syringe containing a slurry of the 10-μm styrene—divinylbenzene copolymer PRP-1 (Hamilton, Reno, NV, U.S.A.) in methanol and pre-swollen 11-μm quaternary ammonium anion-exchange resin Aminex A-28 (Biorad, Richmond, CA, U.S.A.) in aqueous buffer, respectively.

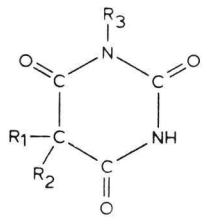
A 100 × 3.0 mm I.D. glass cartridge prepacked with 5-μm Chromspher C_{18} (Chrompack, Middelburg, The Netherlands) served as the analytical column.

Chemicals

Analytical-grade sodium acetate, acetic acid, ammonium acetate, ammonium hydroxide (25%) and HPLC-grade methanol were obtained from J.T. Baker (Deventer, The Netherlands). Pharmaceutical-grade barbiturates were kindly supplied by the Academic Hospital of the Free University; their structures and pK_a values are given in Table I. Stock solutions of the barbiturates (2 mg/ml) were prepared in methanol, stored at 4°C and diluted with an aqueous 0.05 *M* sodium acetate—acetic acid buffer (pH 5.0) prior to use.

TABLE I

STRUCTURES OF THE BARBITURATES USED AS TEST COMPOUNDS



Derivative	R_1	R_2	R_3	pK_a	Supplier
Butobarbital	C_2H_5	*sec.*-Butyl	H	8.0	Brocacef*
Hexobarbital	CH_3	1-Cyclohexenyl	CH_3	8.2	Brocacef
Amobarbital	C_2H_5	3-Methylbutyl	H	7.9	OPG**
Secobarbital	Allyl	1-Methylbutyl	H	7.9	OPG

*Maarssen, The Netherlands.
**Utrecht, The Netherlands.

LC-grade water, prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system, was used for all standard solutions and eluents. The eluents were degassed under vacuum in an ultrasonic bath. The pH of the buffer solutions was adjusted using a Philips

(Eindhoven, The Netherlands) PW 9409 pH meter, either before (acetate buffer; pH 5.0) or after (ammonia—ammonium acetate buffer; pH 9.5) the addition of methanol.

Urine samples were filtered over a 1.2-μm membrane filter and injected directly into the LC system. Their pH should be in the range 4—7.

General procedure

Final experiments were performed using the set-up shown in Fig. 1. The procedure is as follows. A standard barbiturate mixture or a urine sample is transferred by pump A from the 25-μl injection loop to the PRP-1 precolumn using 1 ml of an aqueous 0.05 *M* acetate buffer. Here, sorption of the barbiturates and of various organic contaminants occurs, while inorganic constituents are flushed to waste. Since all anions can interfere with sorption of the barbiturates on the anion-exchange resin, the excess of acetate ions is flushed out using 3.5 ml of a weak ($2 \cdot 10^{-3}$ *M*) aqueous acetate buffer. Next, pump A is switched to deliver 3 ml of $4 \cdot 10^{-3}$ *M* ammonia buffer—methanol (50:50) and the barbiturates, now in their anionic form, are eluted from the PRP-1 precolumn towards the Aminex A-28 anion-exchange precolumn, on which they are trapped as a narrow zone, while neutral organic compounds are flushed to waste. Finally, the barbiturates are neutralized and desorbed by eluting the Aminex precolumn in the forward-flush mode via pump B with 400 μl of 0.1 *M* acetate buffer—methanol (50:50) and separated on the C_{18} analytical column with subsequent UV detection at 220 nm.

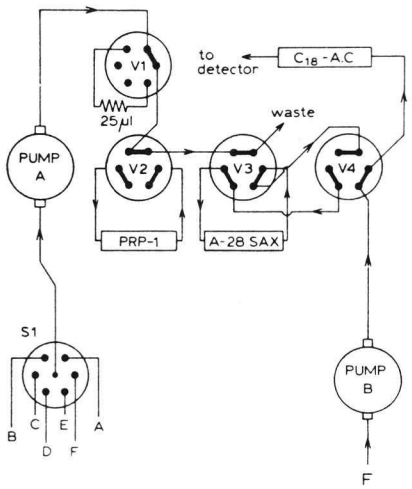


Fig. 1. Experimental set-up for the on-line sample clean-up and determination of barbiturates in urine. S: low-pressure solvent selection valve; V: high-pressure switching valve. A: 0.05 *M* sodium acetate buffer (pH 5.0); B: $2 \cdot 10^{-3}$ *M* sodium acetate buffer (pH 5.0); C: $4 \cdot 10^{-3}$ *M* ammonia buffer (pH 9.5)—methanol (50:50); D: methanol—water (80:20); E: water; F (pump B): 0.1 *M* sodium acetate (pH 5.0)—methanol (50:50). Precolumns: 2 × 4.6 mm I.D. (PRP-1) and 5 × 3.0 mm I.D. (Aminex A-28). Flow-rate of pump A, 2 ml/min (1 ml/min when the ion exchanger was switched in-line).

TABLE II

GENERAL PROCEDURE USING THE SET-UP OF FIG. 1

1. Trapping of the barbiturates on PRP-1 using a 0.05 *M* acetate buffer (pH 5.0)
2. Flushing of PRP-1 with $2 \cdot 10^{-3}$ *M* acetate (pH 5.0)
3. Transfer of barbiturates towards Aminex A-28 with $4 \cdot 10^{-3}$ *M* ammonia buffer (pH 9.5)—methanol (50:50)
4. Flushing Aminex A-28 with ammonia buffer—methanol (50:50)
5. Forward-flush desorption from Aminex A-28 towards the C_{18} analytical column using 0.1 *M* acetate buffer (pH 5.0)—methanol (50:50).
6. Regeneration of PRP-1 with methanol—water (80:20)
7. Flushing both precolumns with water

During the separation step on the C_{18} analytical column, the PRP-1 precolumn is regenerated on-line via pump A with 6 ml of methanol—water (80:20) and, finally, both precolumns are flushed with 3 ml of water.

In order to increase sample throughput, the next run is started while the actual separation is still in progress. The general procedure has been summarized in Table II; details concerning the valve-switching programme will be made available on request.

RESULTS AND DISCUSSION

Characteristics of the precolumns

Breakthrough experiments were carried out according to the procedure described in ref. 9, using a 0.2–1 μg/ml standard solution of butobarbital (pH 5.0). This is the barbiturate showing least retention on the PRP-1 precolumn. At a flow-rate of 2 ml/min the breakthrough volume on the 2 × 4.6 mm I.D. PRP-1 precolumn was found to be 22 ml.

The choice of eluent used for desorption of the barbiturates from the PRP-1 precolumn and their subsequent sorption on the Aminex anion exchanger was found to be rather critical. The use of $4 \cdot 10^{-3}$ *M* ammonia buffer—methanol (50:50) provided both a sufficiently low desorption volume (2.5 ml) for the most highly retained barbiturate, secobarbital, from the PRP-1 precolumn, and sufficient retention of the anionic barbiturates on the Aminex anion exchanger.

TABLE III

GENERAL PERFORMANCE OF THE AUTOMATED BARBITURATE ANALYSIS USING 25-μl STANDARD SOLUTIONS

Compare Table II and the set-up of Fig. 1.

Compound	Repeatability* (n = 11) (% R.S.D.)	Linearity (r)** (n = 7)	Detection limit*** (ng)	Recovery* (%)
Butobarbital	2.5	0.9999	1	95—103
Secobarbital	3.5	0.9998	2	97—105

*Barbiturate level 2 μg/ml.
**Linearity range 0.06—60 μg/ml barbiturate.
***Signal-to-noise ratio = 3:1.

In order to reduce anion competition on the Aminex as much as possible, ammonium acetate instead of ammonium chloride was used; the pH was kept at 9.5. Although at this pH 1—5% of the barbiturates are not dissociated, rapid adjustment of the dissociation equilibrium will prevent any noticeable loss.

It should be mentioned here that retention on the anion exchanger appeared to be highly dependent on flow-rate; the breakthrough volume of butobarbital, in its anionic form, at 0.5 ml/min was almost three times higher than at 1 ml/min (27 vs. 10 ml). We found a breakthrough volume of 10 ml to be amply sufficient for our purposes and used a flow-rate of 1 ml/min in all further experiments.

A 0.1 *M* acetate buffer was found to be satisfactory for the efficient and quantitative desorption of barbiturates from the anion exchanger towards the C_{18} analytical column by < 250 µl of eluent at 0.4 ml/min. In order to avoid hydrophobic interaction on the organic matrix of the anion-exchange resin, and to provide an adequate elution profile on the C_{18} analytical column, the aqueous acetate buffer was diluted with an equal volume of methanol.

Performance and application

With the experimental set-up shown in Fig. 1 and the general procedure described in Table II, analytical data were collected for butobarbital and seco-

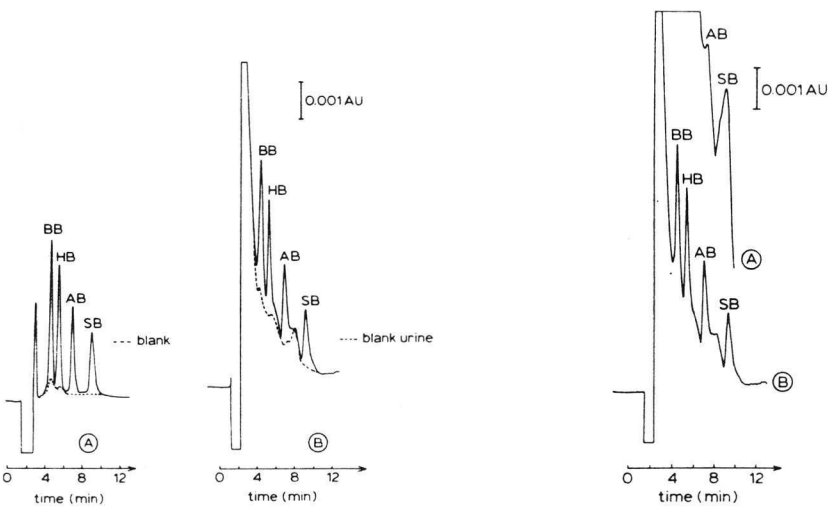


Fig. 2. Chromatograms of 25 µl of (A) a 0.05 *M* acetate buffer and (B) a urine sample, both either with (——) or without (- - - -) barbiturates (spiking level, 1 µg/ml). For procedure, see the set-up of Fig. 1 and Table II. Detection by UV at 220 nm; 0.01 a.u.f.s. Peaks: BB = butobarbital; HB = hexobarbital; AB = amobarbital; SB = secobarbital. Other conditions as in Fig. 1.

Fig. 3. (A) Chromatogram obtained after loading of a 25-µl spiked urine sample on a 2 × 4.6 mm I.D. precolumn packed with PRP-1 using a flush of 6 ml of a 0.05 *M* acetate buffer (pH 5.0) and direct elution with the eluent (F, pump B; cf. Fig. 1) to the analytical column. Other conditions as in Fig. 2. (B) Same chromatogram as in Fig. 2B. Peaks: BB = butobarbital; HB = hexobarbital; AB = amobarbital; SB = secobarbital.

barbital. The results, which are presented in Table III, clearly demonstrate the potential of the method. One should note that, in spite of the repeated sorption/desorption process, the recovery is rather close to 100%.

In Fig. 2, chromatograms for a blank, a standard solution, and a spiked and blank urine sample are shown. Repeated injection of spiked urine samples showed a good repeatability for all four test solutes, as can be seen from Table IV. The recoveries relative to those for standard samples are also satisfactory. Some minor interferences show up in Fig. 2B; however, these are not expected to influence the accuracy of the barbiturate determination, because the barbiturate concentrations will at least be ten-fold higher in real samples as compared to the spiking level in the present experiment. In addition, recoveries are close to 100%.

TABLE IV

APPLICATION OF THE AUTOMATED BARBITURATE ANALYSIS TO 25-μl SPIKED URINE SAMPLES

Compare Table II and Fig. 1; spiking level, 1 μg/ml.

Barbiturate	n	Recovery* (%)	Repeatability (% R.S.D.)
Butobarbital	5	95	4.5
Hexobarbital	4	97	1.1
Amobarbital	4	98	1.0
Secobarbital	5	91	3.2

*Relative to the recovery of standard samples as given in Table III.

The dramatic gain in selectivity obtained by the dual-precolumn approach as compared to a single-precolumn clean-up is demonstrated in Fig. 3. Chromatogram A was obtained after loading the PRP-1 precolumn with 25 μl of spiked urine, flushing with 6 ml of 0.05 *M* acetate buffer, and direct elution of the barbiturates from the precolumn with 0.1 *M* acetate buffer—methanol (50:50) to the analytical column. Chromatogram B was obtained using the dual-precolumn technique. As a consequence of the high selectivity of the latter method, the detection limits in urine are of the same order of magnitude as with standard solutions, i.e. 1—2 ng for 25-μl loop injections.

CONCLUSIONS

Using two sample clean-up precolumns in series, with cleaning action being based on the removal of, first, inorganic and, then, organic compounds, urine samples can be analysed for barbiturates with such selectivity that simple UV detection at 220 nm can be achieved. Modification of the method according to the procedure outlined in ref. 6, which allows detection at 254 instead of 220 nm, may well produce even cleaner chromatograms. The present method, which is fully automated, is linear over at least three orders of magnitude and displays good repeatability. Recoveries in urine are close to 100% at therapeutic concentrations.

In principle, apart from barbiturates, many other biologically active acidic compounds can also be determined with the present method, provided their pK_a values are in the range 1—10, simply by adjusting the pH of the buffer solution that serves as transfer eluent from the first precolumn to the second one. Because of the peak compression that occurs upon transfer from the PRP-1 to the Aminex A-28 precolumn, one is not limited to compounds that display large breakthrough volumes on the PRP-1 precolumn. If necessary, a larger PRP-1 precolumn can be chosen, as was demonstrated earlier in the determination of phenol [7].

Finally, substitution of the anion exchanger by a cation-exchange resin should allow basic compounds to be analysed in a manner similar to the one reported here. This will provide an alternative to the approach of ref. 10, where inorganic interferences were precipitated off-line and organic contaminants filtered out on-line using a PRP-1 precolumn.

ACKNOWLEDGEMENTS

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REFERENCES

1 Martindale, The Extra Pharmacopoeia, The Pharmaceutical Press, London, 27th ed., 1979.
2 R.N. Gupta, J. Chromatogr., 340 (1985) 139.
3 H. Heusler, J. Chromatogr., 340 (1985) 273.
4 R.N. Gupta, P.T. Smith and F. Eng, Clin. Chem., 28 (1982) 1772.
5 C.R. Clark and J.-L. Chan, Anal. Chem., 50 (1978) 635.
6 H. Jansen, C.J.M. Vermunt, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr., 366 (1986) in press.
7 M.W.F. Nielen, J. de Jong, R.W. Frei and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 25 (1986) 37.
8 C.E. Goewie, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 301 (1984) 325.
9 C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 53 (1981) 2072.
10 M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 317 (1984) 557.

Chapter 4. Metal-loaded precolumns

4.1 *Use of a precolumn packed with mercury(II)-8-hydroxyquinoline for the selective on-line trace enrichment of 2-mercaptobenzimidazole in liquid chromatography*

SUMMARY

A small pre-column packed with a mercury(II)–8-hydroxyquinoline phase is used for the selective trace enrichment and clean-up of 2-mercaptobenzimidazole, a thiol model compound. Strong covalent bond formation between the analyte and the metal-loaded sorbent allows the introduction of a wash step with methanol–water (1:1). This results in a superior selectivity to that observed with the frequently used reversed-phase packing materials.

Desorption of the pre-concentrated thiol from the pre-column is effected with an eluent containing cysteine as a displacer. The elution profiles are inefficient, however, so that peak compression of the analyte on the top of the C_{18} separation column is necessary. With UV absorbance detection at 254 nm, the detection limit is 1 ppb. The excellent selectivity of the on-line pre-column sample handling is demonstrated in the analysis of river water and industrial waste water samples.

INTRODUCTION

Sample pre-treatment based on liquid–solid sorption techniques has been shown[1] to be very useful for the trace enrichment of environmental samples in column liquid chromatography (LC). In our group, C_{18}-modified silica[2], styrene–divinylbenzene copolymers[3] and carbon-based[4] sorbents have been used for on-line trace enrichment of many non-polar and moderately polar solutes from aqueous samples, utilizing pre-columns with geometrical volumes of 30–80 μl. For the efficient trace enrichment of highly polar compounds such as polar anilines and phenol, ion-exchange sorbents have been used[5,6]. When using pre-columns packed with reversed-phase materials, however, together with the analyte many other interfering compounds will usually also be concentrated. The selectivity then has to be provided during the detection step, utilizing, *e.g.*, reaction detectors.

As an alternative, we have used[5,6] ion-exchange resins as pre-column packing materials in order to increase the selectivity during the sample handling step. In

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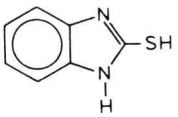

Fig. 1. Structure of 2-mercaptobenzimidazole.

addition, metal-loaded sorbents have been shown to have good potential for the selective sorption of complexing species[7]. Unfortunately, until now the application of these metal-loaded sorbents has been successful only in the selective removal (sorption) of interfering compounds; efficient sorption plus desorption for on-line analysis has been problematic.

From the literature it is known[8–10] that thiol compounds can form very strong covalent mercaptide bonds with mercury(II). Once a thiol has been sorbed on a mercury-loaded phase, several organic–water solvent mixtures can be utilized as flushing solvents to increase the selectivity and clean-up, without seriously affecting the mercaptide bond strength, *i.e.*, the retention of the analyte.

In this study, we immobilized mercury(II) on a commercially available 8-hydroxyquinoline (oxine)-modified hydroxyalkylmethacrylate gel, Spheron oxine, which has a strong affinity for heavy metal ions (but not for the ubiquitous alkali and alkaline earth metal ions), with distribution constants of the order of 10^5 (ref. 11). Retention of mercury(II) on an oxine phase will occur[12] at pH > 4.0. With Spheron oxine, the capacity for several metal ions at pH 5.0 is reported[11] to be 0.1–0.4 mmol g^{-1}, loading with metals being complete within 5–10 min. Alkaline media should be avoided in order to prevent the formation of (hydr)oxides of mercury(II).

2-Mercaptobenzimidazole (Fig. 1) was chosen as a model analyte because of its thiol functionality and its chromophore, which allows simple, although non-selective, UV absorbance detection. This imidazole can occur in the environment as a consequence of its application as a corrosion inhibitor[13] for several metals and as an antioxidant in tyres (and other rubber products). However, as a result of toxicological studies[14] it is considered to be a potential carcinogen, and a method for its selective trace determination in industrial waste water and surface water is therefore of interest.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of a Model 410 pump and a Uvikon 720 LC UV absorbance detector operated at 254 nm was used in combination with a Gilson (Villiers le Bel, France) Model 302 pump, two home-made six-port switching valves and a Rheodyne (Berkeley, CA, U.S.A.) Model 5011 six-way solvent selection valve. Chromatograms were analogue recorded on a Kipp & Zonen (Delft, The Netherlands) BD 40 recorder and processed manually.

Stationary phases and columns

Trace enrichment was carried out on a slightly modified 4 × 2.0 mm I.D.

Chrompack (Middelburg, The Netherlands) pre-concentration column. The pre-column was hand-packed[15] by using a syringe filled with a slurry of 25–40 μm Spheron oxine 1000 (Lachema, Brno, Czechoslovakia) in water–methanol (1:1). For reasons of comparison, several experiments were performed using a home-made 2 × 4.6 mm I.D. pre-column[15] packed with the spherical 10 μm styrene–divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, U.S.A.). The analytical column was a 250 × 4.6 mm I.D. stainless-steel column pre-packed with 5-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.).

Chemicals

HPLC-grade methanol and analytical reagent grade sodium acetate, acetic acid, mercury(II) acetate and mercury(II) chloride were obtained from J. T. Baker (Deventer, The Netherlands) and cysteine from Merck. 2-Mercaptobenzimidazole was a gift from Dr. F. Iverson (Health Protection Branch, Ottawa, Canada). Demineralized water was purified in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system to obtain LC-grade water for use in eluents and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum prior to use.

Preparation of the mercury(II)-loaded sorbent

Spheron oxine (1 g) was suspended in 20 ml of a 0.1 *M* acetate buffer (pH 5.0), 320 mg (*ca.* 1 mmol) of mercury(II) acetate were added and the mixture was mechanically shaken for 2 h. The mercury-loaded phase was collected on a glass filter and washed with acetate buffer (pH 5.0), LC-grade water and methanol. Finally it was dried under vacuum and stored at room temperature.

Procedures

Stock solutions of 2-mercaptobenzimidazole were prepared by weighing and dissolving in methanol, and stored at −20°C. The solutions were diluted with LC-grade water to obtain standard solutions at the (sub)-ppb level. Thiols are easily oxidized to disulphides[16]. Solutions of the model compound and of cysteine therefore had to be degassed in an ultrasonic bath, purged with nitrogen and stored in the dark; in addition, fresh dilutions were prepared daily.

Breakthrough curves of 2-mercaptobenzimidazole on the mercury(II)-loaded phase were recorded according to the procedure reported in ref. 2 using 250 ppb standard solutions in water–methanol (4:1) and a flow-rate of 1 ml min^{-1}.

The river and waste water samples were filtered over a 0.8 μm membrane filter prior to their loading onto the mercury(II)–oxine phase.

RESULTS AND DISCUSSION

Characteristics of the mercury(II)–oxine precolumn

Retention. The retention of a thiol such as 2-mercaptobenzimidazole on the mercury(II)–oxine phase tested will be governed by a least two different mechanisms. Firstly, strong complexation will occur between the mercury ion and the SH group of the test solute. Second, the aromatic rings of the Spheron oxine may be expected to display a distinct hydrophobic interaction towards the phenyl ring of the benzimidazole.

In this retention study, we always prepared the sample solution with 30% methanol. That is, trace enrichment was carried out under conditions in which retention will be predominantly due to covalent bond formation between the thiol group and the divalent mercury. In this situation, the breakthrough volume of 2-mercaptobenzimidazole on a 4 × 2 mm I.D. pre-column was found to be more than 100 ml, which was the maximum sample volume tested.

Desorption. Elution of thiols from a mercury(II)-loaded phase is believed to occur only under one of the following conditions[8]:

(1) the use of a displacer such as another thiol which has a stronger affinity for Hg(II) than the thiol under investigation and/or is present in excess;

(2) the use of a strong mineral acid such as 0.1 *M* hydrochloric acid to break the mercaptide and the mercury(II)–oxine bond;

(3) the use of an Hg(II)-containing solution which will compete with the immobilized mercury for the thiol compound.

We preferred to use a thiol displacer rather than the aggressive hydrochloric acid or the toxic mercury(II) salt solution. In the literature, cysteine and mercaptoethanol have been suggested as effective displacers in off-line applications of an organomercurial–agarose phase[9]. For practical reasons (odour!), we preferred to work with cysteine and used it in all desorption studies.

Desorption from the mercury(II)-loaded pre-column with subsequent on-line transfer to a C_{18} analytical column with water–methanol (3:1) containing 0.05 *M* cysteine was found to result in a huge peak at the start of the chromatogram, which was caused by the Hg(II)–cysteine complex. The imidazole could be desorbed by a 0.5 ml plug of 0.1 *M* cysteine and eluted with a mobile phase containing water–methanol (3:7) without interferences from the early eluting peak, but the peak profile was poor. Although the use of back-flush instead of forward-flush elution slightly improved the situation, we preferred to reconcentrate the broad 2-mercaptobenzimidazole peak on a second, 4 × 4.6 mm I.D., pre-column, packed with C_{18}-modified silica in order to remove the interfering Hg(II)–cysteine complex and to obtain peak compression prior to the actual separation on the C_{18} analytical column.

The suggested approach was only partly successful. 2-Mercaptobenzimidazole indeed eluted as a very narrow peak with a retention time of only 5 min, and without any serious interference from the Hg(II)–cysteine complex. Unfortunately, however, the recovery was only 30–40%, which was due to a partial breakthrough on the second pre-column. Replacing the C_{18} phase in this pre-column by PRP_1 was not sufficient to increase the recovery towards 100%. We therefore decided to apply the peak compression principle on the analytical column itself in all further experiments. This approach turned out to be fully satisfactory (for details, see *Final procedure*).

Regeneration. After a single desorption with cysteine, it was impossible to preconcentrate a second sample on the same mercury(II)–oxine pre-column. Obviously, the divalent mercury had been removed or deactivated by the displacer.

We attempted to perform an on-line regeneration by pumping 10 ml of 0.05 *M* mercury(II) acetate solution through the pre-column. Unfortunately, however, we observed a decreasing recovery of the test solute, probably due to bonding with mercury(II) oxide deposited in the pump, switching valve and/or capillaries of the LC system. Regeneration with 3 ml of 0.05 *M* mercury(II) acetate (or chloride) solution, introduced via a plastic syringe, eliminated the problems and the subsequent

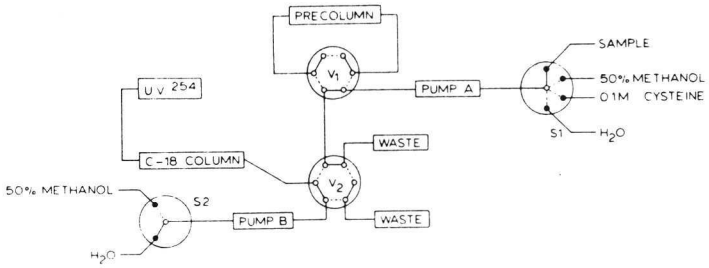

Fig. 2. Experimental set-up for the selective on-line trace enrichment of 2-mercaptobenzimidazole from water samples. S, Low-pressure solvent selection valve; V, high-pressure switching valve. Pre-column, 4 × 2.0 mm I.D. mercury(II)–oxine. Analytical column, 250 × 4.6 mm I.D. packed with 5 μm LiChrosorb RP-18. Eluents as indicated. Flow-rate of pump B, 1 ml min^{-1}. Flow-rate of pump A, as in final procedure.

recoveries now became constant and were comparable to those obtained with fresh mercury(II)–oxine pre-columns. Owing to the obvious difficulty of automating such a regeneration system[7], we recommend the use of an automated cartridge exchange device, such as the Advanced Automated Sample Processor (AASP) (Varian, Sunnyvale, CA, U.S.A.) for metal-loaded sorbents.

Final procedure

The selective on-line trace enrichment of 2-mercaptobenzimidazole was carried out with the experimental set-up shown in Fig. 2. The following procedure was adopted:

(1) 10 ml of aqueous sample solution are loaded onto the mercury(II)-loaded pre-column using pump A at 2 ml min^{-1};

(2) after the sample introduction, the pre-column is flushed with 3 ml of water–methanol (1:1) in order to selectively eliminate interferences retained by a hydrophobic mechanism;

(3) the methanol is removed with 5 ml of LC-grade water;

(4) the analyte is eluted from the pre-column towards the C_{18} analytical col-

TABLE I

ANALYTICAL DATA FOR THE SELECTIVE ON-LINE TRACE ENRICHMENT OF 2-MERCAPTOBENZIMIDAZOLE

Conditions: 10-ml sample solutions analysed according to the final procedure (*cf.*, Fig. 2); data based on peak-area measurements.

Criterion	*Level*	*Result*
Repeatability	11 ppb (n = 10)	±2.6%
Recovery	16 ppb (n = 10)	97%
Linearity (r)	0.5–500 ppb (n = 7)	0.9994
Detection limit	Signal-to-noise ratio = 3:1	10 ng or 1 ppb

umn in the forward-flush mode with 5 ml of 0.1 *M* cysteine in LC-grade water via pump A at 1 ml min^{-1};

(5) the C_{18} analytical column is flushed with 10 ml of LC-grade water via pump B at 1 ml min^{-1} in order to remove the excess of Hg(II)–cysteine;

(6) separation is performed with a step gradient from water to water–methanol (1:1), and 2-mercaptobenzimidazole is detected by UV absorbance at 254 nm.

In order to achieve on-column peak compression in step 4, the C_{18} analytical column has to be conditioned with 9 ml of LC-grade water via pump B at 1 ml min^{-1} prior to the introduction of the pre-column eluate. Fortunately, this conditioning step can be performed during the sample handling on the pre-column (steps 1–3), so the total analysis time is only 28 min. In this study, the experiments were carried out manually, and owing to a lack of an automated cartridge exchange device, a new pre-column cartridge was inserted by hand after each analysis. The entire procedure can, however, easily be automated using a simple microprocessor and a time-based column- and solvent-switching programme, as demonstrated previously[5].

General performance

Table I summarizes the analytical data obtained by means of the final procedure using the set-up in Fig. 2. The recovery was calculated by peak-area comparison with a 100 μl loop injection directly on to the C_{18} analytical column. It should be noted that because of the large breakthrough volume of over 100 ml, the concentration sensitivity can easily be increased by one order of magnitude by introducing larger sample volumes.

Application to real samples

Fig. 3 shows chromatograms of (a) an LC-grade water standard solution, (b) a surface water sample from the river Amstel (Amsterdam, The Netherlands) and (c)

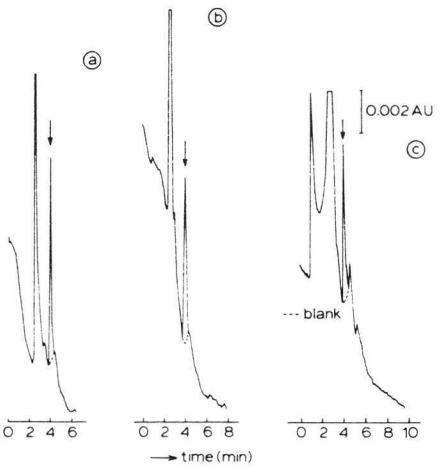

Fig. 3. Chromatograms of 10-ml water samples without and spiked with 11 ppb of 2-mercaptobenzimidazole, analysed using the set-up in Fig. 2 and the final procedure described in the text. (a) LC-grade water; (b) river Amstel water; (c) industrial waste water. Detection at 254 nm, 0.02 a.u.f.s. Other conditions as in Fig. 2.

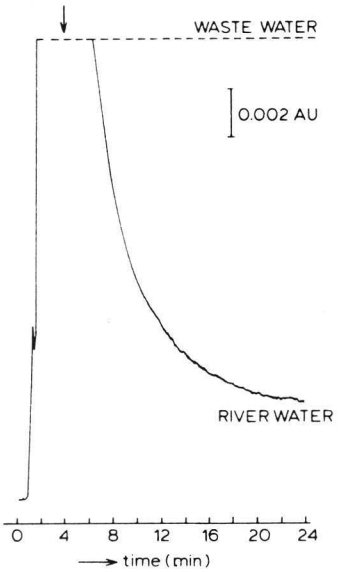

Fig. 4. Chromatograms of 10 ml of the same spiked river and waste water samples (11 ppb of 2-mercaptobenzimidazole) as used in Fig. 3b and c, obtained after on-line trace enrichment on a 2 × 4.6 mm I.D. pre-column packed with PRP_1, flushing with 10 ml of LC-grade water and direct elution to the analytical column with water–methanol (1:1). Other conditions as in Fig. 3.

an industrial waste water sample. Results for the blanks and samples spiked with 11 ppb of 2-mercaptobenzimidazole are given. The early eluting peaks (t_r = 0–3 min) are mainly due to Hg(II)–cysteine, which is not completely eluted before the step gradient (see above). 2-Mercaptobenzimidazole elutes 4 min after the introduction of this step gradient. The selectivity of the method is evident when we consider the close similarity of the chromatograms obtained for the surface and waste water samples and for the LC-grade water sample. The recovery of the test solute in spiked river and waste water compared with that in LC-grade water was 100%.

The good selectivity of the proposed method is also well demonstrated by comparing Figs. 3 and 4. The latter represents the on-line trace enrichment of the same spiked river and waste water samples on a 2 × 4.6 mm I.D. pre-column, packed with the non-selective, highly hydrophobic PRP_1, with an additional flush step with 10 ml of LC-grade water. In this instance, retention was only due to hydrophobic interaction and the pre-column could be eluted with water–methanol (1:1) onto the C_{18} analytical column. However, there is now a definite lack of selectivity, which illustrates the urgent need for the development of selective sorbents for on-line trace enrichment in reversed-phase LC.

CONCLUSIONS

Selective on-line trace enrichment of thiol compounds can be carried out on

small pre-columns packed with mercury(II)-loaded sorbents. Elution has to be carried out using a thiol displacer such as cysteine, with subsequent re-concentration using the on-column peak compression principle because of the poor elution profile.

The excellent selectivity shows up when real samples are analysed and the results are compared with those obtained by a conventional non-selective trace enrichment on a reversed-phase pre-column. The present method is linear over three orders of magnitude and allows the detection of 2-mercaptobenzimidazole in environmental samples at the low parts per billion level with a recovery of almost 100%. Future experiments should prove the general usefulness of the mercury(II)–oxine phase for the selective isolation of other sulphur-containing compounds such as pesticides, drugs and metabolites.

An evaluation of several packing materials loaded with different metal ions is currently being carried out with special emphasis on silver(I)-loaded pre-columns to be used for the selective on-line trace enrichment of compounds containing an acetylenic bond.

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REFERENCES

1 R. W. Frei and U. A. Th. Brinkman, *Trends Anal. Chem.*, 1 (1981) 45.
2 C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 53 (1981) 2072.
3 C. E. Werkhoven-Goewie, W. M. Boon, A. J. J. Praat, R. W. Frei, U. A. Th. Brinkman and C. Little, *Chromatographia*, 16 (1982) 53.
4 W. Golkiewicz, C. E. Werkhoven-Goewie, U. A. Th. Brinkman, R. W. Frei, H. Colin and G. Guiochon, *J. Chromatogr. Sci.*, 21 (1983) 27.
5 M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 317 (1984) 557.
6 M. W. F. Nielen, J. de Jong, R. W. Frei and U. A. Th. Brinkman, *Int. J. Environ. Anal. Chem.*, (1986) in press.
7 C. E. Goewie, P. Kwakman, R. W. Frei, U. A. Th. Brinkman, W. Maasfeld, T. Seshadri and A. Kettrup, *J. Chromatogr.*, 284 (1984) 73.
8 Z. Slovak, M. Smrz, B. Docekal and S. Slovakova, *Anal. Chim. Acta*, 111 (1979) 243.
9 *Bulletin 2066*, Bio-Rad Labs., Richmond, CA, 1982.
10 C. S. Russell and S. E. Pollack, *J. Chromatogr.*, 166 (1978) 632.
11 Z. Slovak, S. Slovakova and M. Smrz, *Anal. Chim. Acta*, 75 (1975) 127.
12 F. Vernon and H. Eccles, *Anal. Chim. Acta*, 63 (1973) 403.
13 K. P. Soni and I. M. Bhatt, *J. Electrochem. Soc. India*, 32(2) (1983) 197.
14 A. Korzhohen, *Arch. Environ. Contam. Toxicol.*, 11 (1982) 753.
15 C. E. Goewie, M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 301 (1984) 325.
16 S. Patai, Editor, *The Chemistry of the Thiol Group*, Wiley, New York, 1974, p. 785.

4.2 *Metal-loaded sorbents for selective on-line sample handling trace enrichment in liquid chromatography*

ABSTRACT

Three different commercially available stationary phases containing a thiol, an 8-hydroxyquinoline (oxine), and a 2-amino-1-cyclopentene-1-dithiocarboxylic acid (ACDA) functional group, were loaded with mercury (Hg(II)), platinum (Pt(IV)) and silver (Ag(I)) ions. The phases were packed in small precolumns and evaluated for their potential towards the selective on-line sample handling and trace enrichment of three model systems in liquid chromatography. The thiol 2-mercapto- benzimidazole was used to study trace enrichment on Hg(II)-loaded phases; the herbicide buturon, which contains an ethynic bond, was selected to study trace enrichment on Ag(I)-loaded phases. The on-line filter effect of Pt(IV)-loaded phases was investigated with 4-chloroaniline as a model compound.

The results indicate that the Hg(II)-ACDA phase should be preferred for the trace enrichment of thiols, a Pt(IV)-ACDA or a Pt(IV)-thiol phase for the anilines and the Ag(I)-oxine phase for the trace enrichment of ethynic compounds.

As environmental application, the selective on-line trace enrichment of buturon from river water samples, on a precolumn packed with Ag(I)-oxine, is shown.

INTRODUCTION

Ligand-exchange phenomena (1) have been used for the liquid chromatographic separation of compounds of environmental and biomedical interest. Kettrup et al. described chelating resins (2,3) and a silica-based 2-amino-1-cyclopentene-1-dithio-carboxylic acid (ACDA) phase (4) and investigated the sorption and elution behaviour for several metal ions. Takayanagi et al. (5) loaded an ACDA phase with either copper or silver ions for the separation of dialkyl sulphides. Iminodiacetate phases were loaded with nickel (6), zinc (7) and copper (8) for the separation of peptides, amino acids and proteins.

So far, only a few authors have used the potential of ligand-exchange phenomena for selective sample handling purposes. Veuthey et al. (9) developed a bis-dithio-carbamate-copper and a cyclam-copper phase and demonstrated their applicability for the on-line preconcentration of amino and carboxylic acids. Andersson et al. (10) used a

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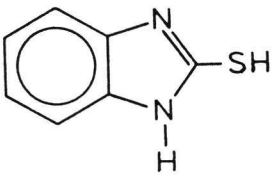
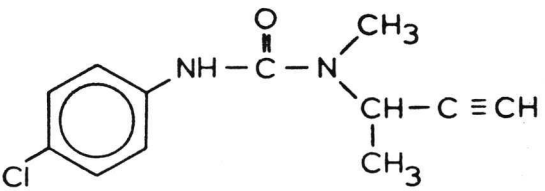
cation-exchanger in its silver form for the selective isolation of ethynyl steroids from urine and our group applied a Pt(IV)-ACDA phase to the selective removal of interfering anilines from a phenylurea herbicide mixture (11). More recently, we used a precolumn packed with Hg(II)-oxine for the selective on-line trace enrichment of 2-mercaptobenzimidazole (12). Excellent selectivity, even when using ordinary UV absorbance detection at 254 nm, was demonstrated at low-ppb levels.

The aim of the present study was to evaluate various commercially available packings, e.g., an ACDA, an oxine and a thiol phase for the selective on-line sample handling and trace enrichment of three model systems: 2-mercaptobenzimidazole



on Hg(II)-loaded phases, 4-chloroaniline on Pt(IV)-loaded phases and buturon



(a herbicide containing an ethynic bond) on a Ag(I) phase.

Special attention was given to the Ag(I)-loaded phases, which were studied in more detail because of the relatively low complexation constants of Ag(I)-ethynic bonds. In addition, the environmental applicability of these Ag(I) phases will be shown.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps was used in combination with a Perkin-Elmer (Norwalk, CT, U.S.A.) LC-75 variable-wavelength UV absorbance detector, a home-made six-port switching valve and a Rheodyne (Berkeley, CA, U.S.A.) Model 5011 six-way solvent selection valve. Breakthrough curves and chromatograms were analog recorded on a Kipp & Zonen (Delft, The Netherlands) BD 8 recorder and processed manually.

Stationary phases and columns

8-Hydroxyquinoline-modified hydroxyalkyl-methacrylate gel, Spheron Oxine 1000 (25-40 μm), and thiol-modified hydroxyalkyl-methacrylate gel, Spheron Thiol 1000 (40-63 μm) were obtained as a gift from Lachema (Brno, Czechoslovakia). 2-Amino-1-cyclopentene-1-dithiocarboxylic acid-modified silica, ACDA (40 μm) was synthesized on request and donated by Analytichem (Harbor City, CA, U.S.A.). The structures of these stationary phases are given in Fig. 1.

Trace enrichment was carried out on 2 x 4.6 mm I.D. home-made precolumns (13) which were hand-packed with the metal-loaded (see below) stationary phase with a microspatula and using a thick slurry of the packing material in methanol.

For reasons of comparison, some experiments were performed using 40 μm octyl-bonded silica (Baker, Deventer, The Netherlands).

The analytical column was a 100 x 3.1 mm I.D. stainless-steel column home packed with 10 μm Spherisorb ODS-2 (Phase Separations, Queensferry, U.K.).

Chemicals

HPLC-grade methanol, HPLC-gradient grade acetonitrile and analytical-grade sodium acetate, acetic acid, mercury(II) acetate, sodium chloride, sodium hydroxide, nitric acid and silver(I) acetate were obtained from Baker; cysteine was obtained from Merck (Darmstadt, F.R.G.) and EDTA from Sigma (St. Louis, MO, U.S.A.).

4-Chloroaniline was purchased from Fluka (Buchs, Switzerland); buturon was a gift from the Food Inspection Service (Amsterdam, The Netherlands) and 2-mercaptobenzimidazole was a gift from Dr. F. Iverson (Health Protection Branch, Ottawa, Canada).

Demineralized water was purified in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system to obtain LC-grade water for use in eluents and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum prior to use.

Preparation of the metal-loaded sorbents

1 g of Spheron Oxine, Spheron Thiol or ACDA-bonded silica was suspended in 25 ml of a 0.05 M Hg(II) acetate solution in LC-grade water and each mixture was mechanically shaken for 2 h. The Hg(II)-loaded phases were collected on glass filters and washed with 30 ml of LC-grade water and dried for 2 h at 100°C.

Ag(I)-loaded phases were prepared in a similar way by using a 0.05 M Ag(I) acetate solution in 1 mM acetic acid. 0.5 g of Spheron Oxine, Spheron Thiol or ACDA-bonded silica was suspended in 25 ml of a 0.025 M sodium

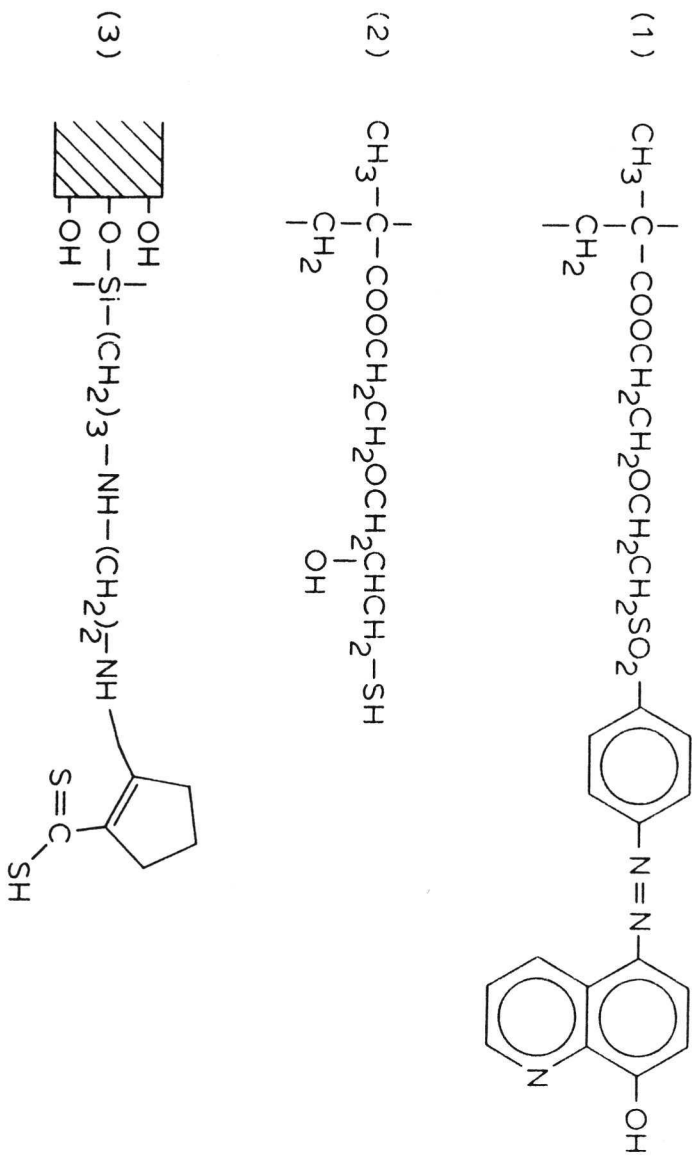


Fig.1. Structure of the sorbents under investigation. (1) 8-Hydroxyquinoline--modified hydroxyalkyl-methacrylate gel, Spheron Oxine; (2) thiol-modified hydroxyalkyl-methacrylate gel, Spheron Thiol; (3) 2-amino-1-cyclopentene-1-dithiocarboxylic acid-modified silica ACDA.

hexachloroplatinate(IV) solution (adjusted to pH 5 with NaOH) and further processed similar to the mercury phases.

Procedures

Stock solutions of the model compounds were prepared by weighing and dissolving in methanol; they were stored at -20°C. The solutions were diluted with methanol-water mixtures to obtain standard solutions at the ppb level. Solutions of 2-mercaptobenzimidazole and cysteine were freshly prepared each day, degassed in an ultrasonic bath, purged with nitrogen and stored in the dark to prevent oxidation to disulphides.

The capacity of the metal-loaded sorbents was calculated by determining the amount of metal ion remaining in solutions and wash solvents after the preparation of the phases. Hg(II) was determined with a complexometric back titration of an excess of EDTA by zinc sulphate (14); Ag(I) was titrated with bromide (15) and Pt(IV) was determined by ICP-AES.

Breakthrough studies were carried out (in triplicate) according to the procedure reported in ref. 16, using 250 ppb standard solutions of the model compounds in water-methanol (7:3), at a flow-rate of 1 ml min^{-1}.

Elution profile studies were carried out following the procedures reported in the Results and Discussion section.

River water samples were filtered over a 0.8 μm membrane filter prior to their trace enrichment onto the Ag(I)-oxine phase.

RESULTS AND DISCUSSION

Capacity

Data concerning the capacity of the various metal-loaded sorbents are summarized in Table I. The preparation of these phases was carried out in triplicate and the results were found to be reproducible within 5-10%. Metal-loaded ACDA-bonded silica provided highest capacity in all cases; however, for Hg(II)- -loaded phases differences are relatively small. Pt(IV) could not be effectively loaded on the oxine phase and the loadability for Ag(I)-thiol is relatively poor. The data for the Pt(IV)-ACDA phases were taken from another study (11) and are not directly comparable. According to these data, no limitations show up for selecting a stationary phase to be loaded with Hg(II). Pt(IV) loading should be performed on either thiol or ACDA stationary phases and Ag(I) preferably on ACDA or oxine phases

TABLE I

Capacity of Metal-Loaded Sorbents (mMol g^{-1})*

Sorbent d_p (μm)	Hg(II)	Ag(I)	Pt(IV)	
Spheron Oxine	25-40	0.31	0.18	0.03
Spheron Thiol	40-63	0.29	0.10	0.16
ACDA-Silica	40	0.40	0.32	0.52**

*For determination, cf. Experimental section.
**Data from ref. 11.

Retention characteristics of the metal-loaded precolumns

The retention of the model compounds on the various metal-loaded phases will be governed by at least two different mechanisms. Firstly, strong complexation will occur between the metal ion and the functional group of the test solute. Secondly, carbon chains and aromatic rings of the stationary phases may be expected to display a distinct hydrophobic interaction with the aromatic system of the model compounds. In retention studies we always prepared the sample solution in water-methanol (7:3). That is, trace enrichment was carried out under conditions in which retention will be predominantly due to ligand-exchange phenomena, while hydrophobic interaction will be suppressed.

It was found that all Hg(II)-loaded phases showed breakthrough volumes of at least 100 ml (the maximum volume tested) for a 250 ppb solution of 2-mercaptobenzimidazole, which does not contradict with the capacity data in Table I.

For Pt(IV)-loaded phases breakthrough curves are shown in Fig. 2. It can be seen that the Pt(IV)-oxine phase is not suitable for the trace enrichment of the model compound; complete breakthrough occurs almost instantaneously due to the low capacity of this phase (cf. Table I). The Pt(IV)-ACDA sorbent shows equal or better performance than the Pt(IV)-modified thiol phase.

Fig. 3 shows breakthrough curves of buturon on the Ag(I)-modified phases. Because of its lower capacity, the Ag(I)-thiol seems to be less suitable. However, contrary to the capacity data, the Ag(I)-oxine phase shows much better retention characteristics towards the model compound than Ag(I)-ACDA.

In general, one should notice the different shape of the breakthrough curves for

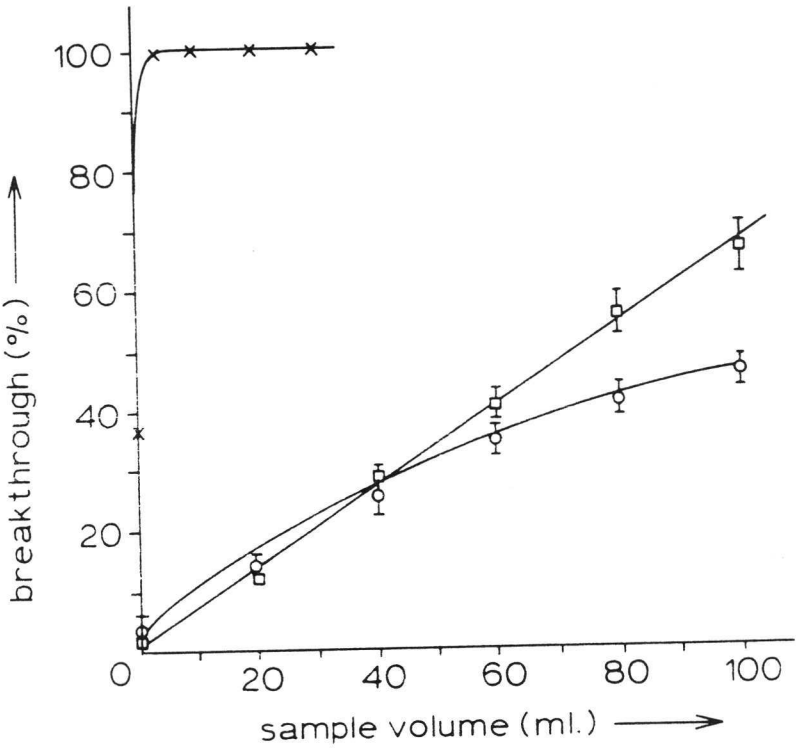


Fig. 2. Breakthrough curves of 4-chloroaniline on Pt(IV)-loaded oxine (x), thiol ( ) and ACDA (o). Conditions: precolumn 2 x 4.6 mm I.D., flow-rate 1 ml min^{-1}; 250 ppb 4-chloroaniline in methanol-water (3:7). Detection at 245 nm.

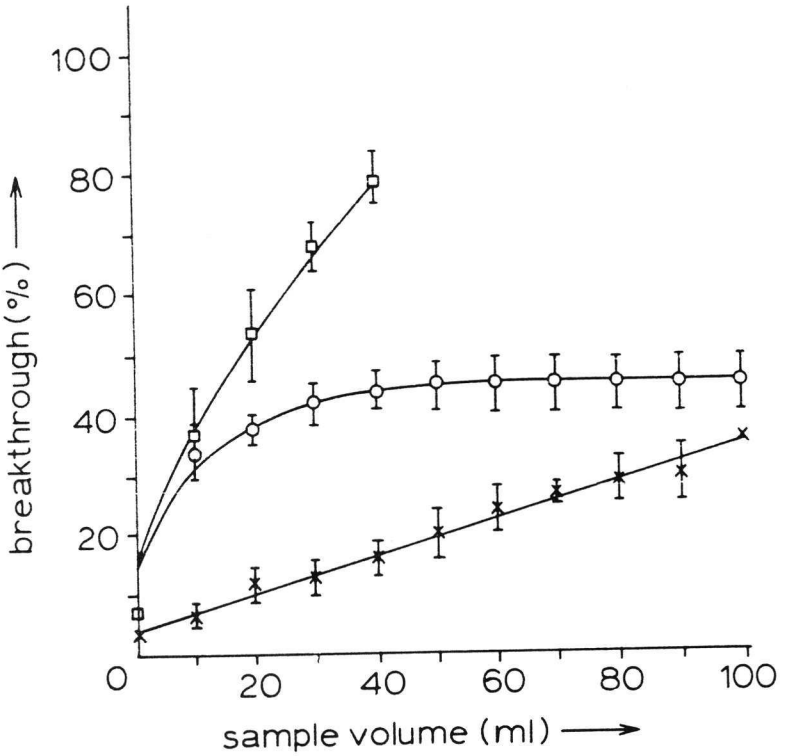


Fig. 3. Breakthrough curves of buturon on Ag(I)-loaded oxine (x), thiol ( ) and ACDA (o). Conditions: 250 ppb buturon in methanol-water (3:7). Other conditions as in Fig. 2.

Pt(IV) and Ag(I) phases as compared to the situation in e.g. ref. 16, where precolumns packed with hydrophobic materials were investigated. A slowly increasing partial breakthrough seems to occur starting with the first ml of sample. However, this was found not to influence the recovery or repeatability in the final application (cf. below). The observed differences are difficult to explain, but it is obvious that more than one complexation mechanism is involved. Metals have more than one coordination number, hence a mixture of different retention strenghts may be expected. Unfortunately, literature data concerning complexation usually refer to homogenic systems, which are not directly comparable with the present situation.

Because of the relatively low complexation constant (17) of the Ag(I)-acetylene bond as compared to, e.g., a Hg(II)-cysteine bond (K = 16 versus 10^{42}), silver phases were studied in more detail.

Retention on Ag(I)-loaded precolumns

Breakthrough curves were recorded using a 25 ppb solution of buturon. The retention characteristics were found to be essentially the same as in Fig. 3, although standard errors for each data point increased, because measurements had to be performed close to the detection limit. These results suggest that overloading is not a dominant factor in the explanation of the retention behaviour.

Fig. 4 shows the influence of the linear velocity on the breakthrough curve of buturon on the Ag(I)-oxine phase. It can be seen that up to 2 mm s^{-1} (ca. 2 ml min^{-1}) the retention is not influenced by the linear velocity. However, the use of a pulse damper connected to the sampling pump, was found to be essential for obtaining reproducible retention on Ag(I)-loaded precolumns.

Strong influences on the retention were found after the addition of 1 mM EDTA to the sample solution: breakthrough now occurred almost immediately. The same effect was found after the addition of saline (0.9% NaCl) to the sample.

One can conclude that in the application of small Ag(I)-loaded precolumns to real environmental and biomedical samples - which usually contain trace metals, saline, added EDTA etc. - several precautions should be taken to prevent insufficient loading due to the presence of, e.g., competing trace metals in the sample, and to prevent overloading of the Ag(I) phase by competing ligands in the sample which may even precipitate (AgCl!) on the precolumn. The bulk of possible interferences is easily eliminated in practice, by using a dual-precolumn approach, as will be demonstrated elsewhere (18).

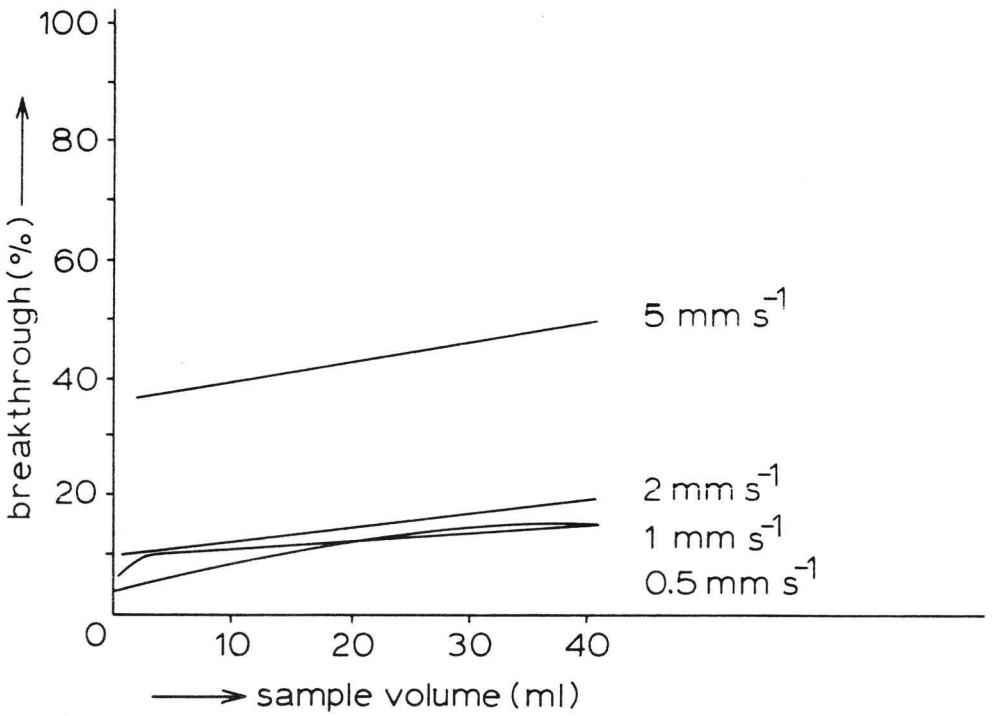


Fig. 4. Breakthrough curves of buturon on Ag(I)-oxine at different linear velocities. Conditions: as in Fig. 3, except for flow-rate.

Elution characteristics of the metal-loaded precolumns

Pt(IV)-loaded precolumns are used for selective filtering only (cf. ref. 11) because efficient elution often requires high concentrations of mineral acids (3,4) which are not directly compatible with on-line sample handling systems. Consequently, we compared the elution profiles for different Ag(I)- and Hg(II)-loaded precolumns only.

In the case of Hg(II)-loaded precolumns, 6-20 ml of a 250 ppb 2-mercaptobenzimidazole solution in methanol-water (3:7) were loaded onto the precolumn followed by a 5 ml flush with methanol-water (3:7) at 1 ml min^{-1}. Elution was performed by a 0.5 ml plug of 0.1 M cysteine in methanol-water (3:7) without reconcentration on the top of the C18 analytical column (contrary to ref. 12), in order to be able to study the contribution of the metal-loaded sorbent to the total band broadening. Each experiment was carried out in triplicate. The results expressed in terms of plate number and asymmetry (at 10% of the peak height), are presented in Table II. Although capacity (cf. Table I) on the various Hg(II) precolumns was found to be comparable, the elution profiles certainly favour the use of the Hg(II)-ACDA phase.

TABLE II

Elution Profiles for Different Hg(II)- and Ag(I)-loaded Precolumns

For conditions, see text.

	Plate number	Asymmetry (0.1 h)
Hg(II)-oxine	100 ± 15	5.8 ± 0.5
Hg(II)-thiol	700 ± 15	3.2 ± 0.2
Hg(II)-ACDA	1600 ± 450	1.7 ± 0.1
Ag(I)-oxine	275	-
Ag(I)-thiol	275	2.4 ± 0.3
Ag(I)-ACDA	-	-

-, Could not be determined.

In the case of Ag(I) precolumns 10 ml of a 10 ppb buturon solution in methanol-water (3:7) was loaded onto the precolumn followed by a 2.5 ml flush at 0.5 ml min^{-1}. Elution to the C18 analytical column was accomplished with a mobile phase

consisting of acetonitrile-water (35:65), acidified with nitric acid to pH 2. From Table II it can be seen that the elution behaviour when using Ag(I)-oxine and -thiol is similar, although retention was found to differ significantly (cf. Fig. 3). Surprisingly, it was found impossible to obtain a buturon peak on the Ag(I)-ACDA phase, which prevents further application of this system. Possibly other, chemical, reactions play a significant role here promoting, e.g., silver sulphide formation. Generally spoken, the plate number of the peaks is not very impressive, which is obviously due to the strong interaction between the sample and sorbent. In practice this problem can be solved by applying peak-compression techniques on top of the C18 separation column, as was earlier demonstrated for 2-mercaptobenzimidazole (12).

Re-use of the metal-loaded precolumns

In order to investigate the possibilities of their re-use, the metal-loaded precolumns were treated with an excess (4 ml) of eluent and flushed with methanol-water (3:7) until baseline stabilization. Then a breakthrough curve was recorded as described under "Retention characteristics".

For all Hg(II)-loaded phases breakthrough occurred immediately. Obviously, the Hg(II) was removed from the sorbent by the cysteine-containing eluent, which is to be expected, considering the complexation constants for Hg(II)-oxine and Hg(II)-cysteine (10^5 and 10^{42}, respectively). The same results were obtained with the Ag(I)-loaded phases after treatment with 4 ml of the acetonitrile-water (35:65) eluent, acidified with nitric acid to pH 2.

The results reported in an earlier paper (11) that elution and regeneration using Pt(IV)-loaded precolumns can be performed by flushing with pure acetonitrile turned out to be too optimistic; we never obtained a satisfactory elution pattern with the present Pt(IV)-ACDA phases. Flushing these Pt(IV)-loaded precolumns with 4 ml of acetonitrile had no influence at all on the retention characteristics of the model compound 4-chloroaniline: exactly the same breakthrough curves were obtained as in Fig. 2. One may conclude that the Pt(IV)-thiol and -ACDA phases can be used for more than one sample; however, due to the absence of an eluent compatible with an on-line system, these platinum filters (11) may get saturated by (other) complexing species. The possibility of re-use will therefore be determined by the complexity and the volume of the samples under investigation.

As outlined in ref. 12, regeneration of metal-loaded precolumns is sometimes possible but in practice it is laborious and difficult to automate. In addition, it will be more economical to repack the small precolumns than to regenerate them with, e.g., an

excess of a metal salt solution. Therefore, for metal-loaded sorbents, we generally recommend the use of an automated precolumn exchange device such as the AASP (Varian, Sunnyvale, CA, U.S.A.) or the automated cartridge exchanger described by Nielen et al. [19].

Application of the Ag(I)-oxine precolumn

Contrary to the Pt(IV) and Hg(II) precolumns (11,12), the Ag(I)-loaded precolumn as yet has not been used for an environmental application.

We used the set-up of Fig. 5. 10 ml of a 10 ppb solution of the herbicide buturon in water were applied to the precolumn via pump A at 0.5 ml min^{-1}. After introduction of the sample, the precolumn was flushed with 2 ml of methanol-water (3:7). Then the valve was switched and the precolumn was eluted by acetonitrile-water (4:6), acidified to pH 2 with nitric acid, at a flow of 0.5 ml min^{-1}. The precolumn was repacked with fresh Ag(I)-oxine material after each analysis. Although this system was operated manually, it has been shown before that it can be easily automated using a microprocessor with a time-based valve- and solvent- switching program (11).

Table III summarizes the analytical data obtained by this procedure. The recovery, calculated by peak area comparison with the mean of four 100 µl loop injections directly onto the C18 analytical column, is in agreement with the 7% loss due to breakthrough, as predicted for a 10 ml sample by Fig. 4.

TABLE III

Analytical Data for the Selective On-line Trace Enrichment of Buturon on a Ag(I)-oxine Precolumn.
Conditions: 10 ml sample solutions analyzed according to the procedure in the text (cf. Fig. 5); data based on peak area measurements.

Criterion	Level	Results
Repeatability	10 ppb (n = 9)	± 3% RSD
Recovery	10 ppb (n = 9)	95 ± 3%
Linearity (r)	1-500 ppb (n = 7)	0.9994
Detection limit	S/N = 3/1	1.5 ng or 0.15 ppb

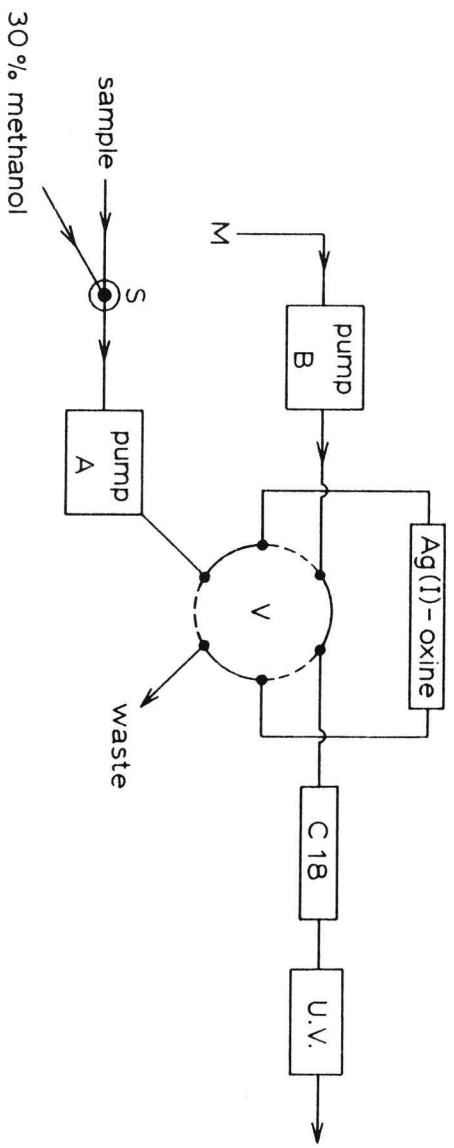


Fig. 5. Experimental set-up for the selective on-line trace enrichment of buturon from water samples. S, low-pressure solvent selection valve; V, high-pressure switching valve. Precolumn 2 x 4.6 mm I.D. Ag(I)-oxine. Analytical column 100 x 3.1 mm I.D. packed with 10 µm Spherisorb ODS-2. M, acetonitrile-water (4:6) acidified to pH 2.0 with nitric acid. Flow rate of pumps A and B, 0.5 ml min^{-1}. Detection at 245 nm, 0.02 AUFS.

Fig. 6 shows chromatograms of (a) an LC-grade water standard solution and (b) a surface water sample (river Waal, Lobith, The Netherlands). Results for the blanks as well as samples spiked with 10 ppb of buturon are given. The selectivity of the ligand-exchange sample handling is already evident when we consider the similarity between the standard and the river water sample. It is even more nicely demonstrated by comparing chromatograms (b) and (c); the latter represents the on-line trace enrichment of the same spiked river water sample on a precolumn packed with non-selective octyl-bonded silica. In this case, retention was only due to hydrophobic interaction and the precolumn could only be flushed with water, which clearly results in a serious lack of selectivity.

The method was found to be selective for the herbicide buturon. After preconcentration of a mixture of nine different phenylurea herbicides, only buturon - being the only analyte to contain an ethynic bond - appeared in the final chromatogram.

CONCLUSIONS

Selective on-line sample handling and trace enrichment based on metal complexation can be successfully carried out using small precolumns packed with metal-loaded stationary phases. Three commercially available stationary phases, having a relatively large particle size and either based on silica or on a soft polymer, were easily and reproducibly loaded with Hg(II), Pt(IV) and Ag(I). After determining the capacity, one could conclude that Pt(IV)-oxine phases should be rejected and Ag(I)-thiol phases should be handled with caution because of their relatively low capacity.

Retention on the metal-loaded precolumns still is difficult to interpret. Breakthrough volumes for the Hg(II)-loaded precolumns were at least 100 ml but the breakthrough curves for the Pt(IV)- and Ag(I)-loaded precolumns were found to have a significantly different shape as compared to curves for precolumns packed with hydrophobic materials. Besides, the retention on Ag(I)-loaded precolumns was strongly influenced by, e.g., EDTA and chloride ion. Because of the very low complexation constant of the Ag(I)-ethynic bond, one should always consider the presence of these and other interferences in real samples.

The elution profiles obtained from metal-loaded precolumns were found to be rather inefficient; peak compression techniques on top of the separation column can, however, as a rule dramatically improve the final result.

On the basis of capacity, retention and elution behaviour, one can recommend a Hg(II)-ACDA phase for the selective trace enrichment of thiol compounds; the selective filtering of anilines should be done by means of a Pt(IV)-loaded ACDA or thiol phase and

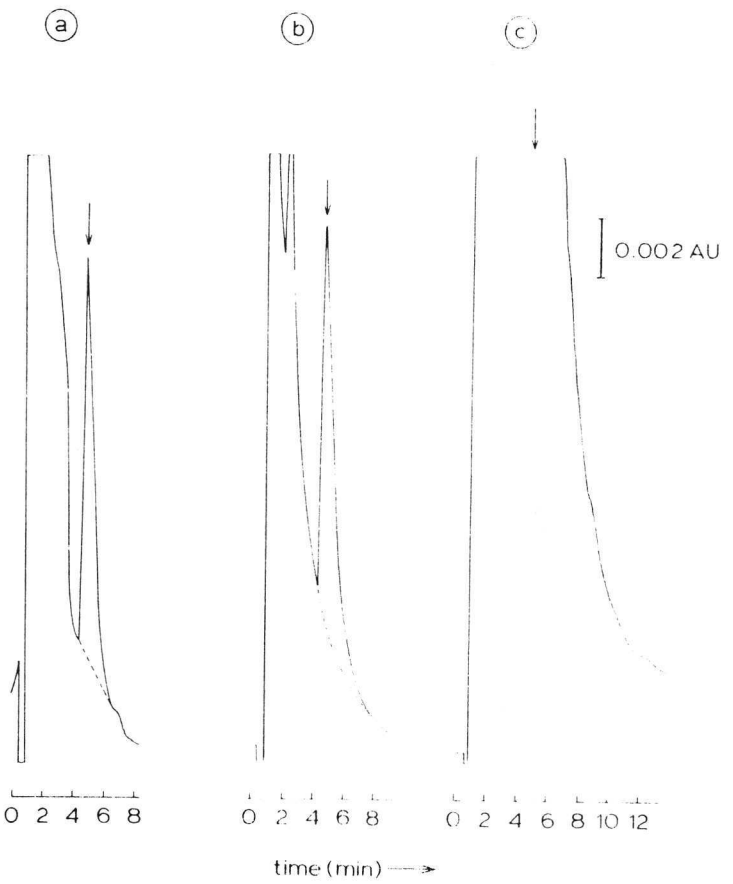


Fig. 6. Chromatograms of 10 ml water samples spiked with 10 ppb of buturon and analyzed using the set-up of Fig. 5 and the procedure described in the text. (a) LC-grade water solution, (b) river Waal water, and (c) river Waal water, preconcentrated on an octyl-bonded silica precolumn. Blanks, ------. Other conditions as in Fig. 5.

a Ag(I)-oxine phase should be recommended for the selective on-line trace enrichment of ethynic compounds.

On-line regeneration of metal-loaded precolumns was found to be laborious. In addition, the use of an excess of a metal salt solution for regeneration is not economical. Fortunately, the metal-loaded precolumns are inexpensive, and consequently they should be used only once. By using an automatic cartridge exchange device, fully automated procedures can be developed.

Although retention, elution and regeneration still present various experimental problems, three interesting applications of selective on-line sample handling and trace enrichment using metal-loaded precolumns have now been demonstrated (refs. 11 and 12, and this paper), one of these featuring the application of the rather labile Ag(I)-ethynic bond formation. Each of them shows good linearity and repeatability, low- or sub-ppb detection limits and an excellent selectivity with real samples even when using non-selective UV detection. This superior selectivity argues for the rapid development and commercialization of more types of selective sorbents for on-line sample handling and trace enrichment in liquid chromatography.

The conversion of an off-line method for the determination of ethynyl steroids in urine using Ag(I)-loaded precolumns (10) into an automated on-line procedure is presently being studied (18).

ACKNOWLEDGEMENTS

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References

1. Davankov, V.A. and Semechkin, A.V., J. Chromatogr., 141, 313 (1977).
2. Seshadri, T. and Kettrup, A., Z. Anal. Chem., 310, 1 (1982).
3. Grote, M. and Kettrup, A., Anal. Chim. Acta, 172, 223 (1985).
4. Grote, M. and Kettrup, A., Anal. Chim. Acta, 175, 239 (1985).
5. Takayanagi, H., Hatano, O., Fujimura, K. and Ando, T., Anal. Chem., 57, 1840 (1985).
6. Hemdan, E.S. and Porath, J., J. Chromatogr., 323, 247 (1985).

7. Kato, Y., Nakamura, K. and Hashimoto, T., J. Chromatogr., 354, 511 (1986).
8. Gimpel, M. and Unger, K., Chromatographia, 17, 200 (1983).
9. Veuthey, J.-L., Bagnoud, M.-A. and Haerdi, W., Int. J. Environ. Anal. Chem., (1986) in press.
10. Andersson, S.H.G., Axelson, M., Sahlberg, B.-L. and Sjövall, J., Anal. Lett., 14, 783 (1981).
11. Goewie, C.E., Kwakman, P., Frei, R.W., Brinkman, U.A.Th., Maasfeld, W., Seshadri, T. and Kettrup, A., J. Chromatogr., 284, 73 (1984).
12. Nielen, M.W.F., Bleeker, R., Frei, R.W. and Brinkman, U.A.Th., J. Chromatogr., 358, 393 (1986).
13. Goewie, C.E., Nielen, M.W.F., Frei, R.W. and Brinkman, U.A.Th., J. Chromatogr., 301, 325 (1984).
14. Vogel, A.I., A Textbook of Quantitative Inorganic Analysis, 3rd ed., Longmans, London, p. 424 (1961).
15. Vogel, A.I., A Textbook of Quantitative Inorganic Analysis, 3rd ed., Longmans, London, p. 80 (1961).
16. Werkhoven-Goewie, C.E., Brinkman, U.A.Th. and Frei, R.W., Anal. Chem., 53, 2072 (1981).
17. Gmelin Handbuch der anorganischen Chemie, 8e Aufl., (B6), Springer-Verlag, Berlin (1975).
18. Nielen, M.W.F., Van Soest, R.E.J., Van Ingen, H.E., Frei, R.W. and Brinkman, U.A.Th., in preparation.
19. Nielen, M.W.F., Valk, A.J., Frei, R.W. and Brinkman, U.A.Th., Chromatographia, submitted for publication (1986).

4.3 Selective on-line trace enrichment for the determination of ethynyl steroids in urine by liquid chromatography with precolumn technology.

INTRODUCTION

Ethynyl steroids are being prescribed extensively as contraceptives, to control menstrual disorders and to suppress menopausal symptoms. Because of the toxicity of estrogens, the daily intake via contraceptive applications is reduced to the absolute minimum and, for ethynylestradiol, typically is in the order of 10-50 μg [1]. Ethynylestradiol is partly excreted unchanged in urine, but this occurs very slowly. Consequently, concentration levels in urine are in the low ng per ml range requiring very sensitive and selective methods for the determination in urine.

Heikkinen et al. [2] described the use of ion-exchange chromatography in steroid analysis prior to gas chromatography (GC) or GC-mass spectrometry (GC-MS). Phenolic steroids were selectively isolated by anion-exchangers and ethynyl steroids by cation-exchangers in the silver(I) form. Tetsuo et al. [3] reported the analysis of ethynyl steroids, using hydrophobic and ion-exchange (in Ag(I) form) precolumns, followed by derivatization and GC-MS. Recoveries for urine spiked with non-conjugated steroids were 80-90% and the detection limit was about 5 pg/ml. Andersson et al. [4] described an improved method for the clean-up of ethynylestradiol from urine. The glucuronides were enzymatically hydrolyzed and the released hormones were trapped on a hydrophobic sorbent. Next the solutes were eluted and selectively reconcentrated on a cation-exchange sorbent in its Ag(I) form. Thanks to the use of a Ag(I)-loaded precolumn the ethynyl steroids could be selectively isolated, even in the presence of relatively high concentrations of endogeneous steroids. The main disadvantage of the quoted methods is the use of many off-line sample manipulations which results in a rather long analysis time. In addition, derivatization is necessary, and expensive GC-MS systems are used.

Recently, we [5] used a small precolumn packed with silver(I)-8-hydroxyquinoline (Ag(I)-oxine) for the selective on-line trace enrichment of a herbicide from river water samples. Buturon, the only phenylurea herbicide having an ethynyl group, is able to interact selectively with the Ag(I)-loaded precolumn. The application of a Ag(I)-loaded precolumn to urine samples is, however, more critical because of the high concentration of interfering compounds, e.g. chloride ions and amino acids. By using a dual-precolumn approach, these interferences may be eliminated in an automated way, as was recently demonstrated in the clean-up of barbiturates from urine [6], viz. via a solid phase extraction procedure involving a hydrophobic sorbent prior to the selective, i.e., Ag(I)-oxine, precolumn.

Liquid chromatographic (LC) methods for the determination of ethynyl steroids in urine are often hindered by the poor resolution between norethindrone, norgestrel and ethynylestradiol. Papas et al. [7] used a ternary mobile phase to obtain sufficient resolution between norethindrone and ethynylestradiol. However, by monitoring at two wavelengths simultaneously, ethynylestradiol can be selectively determined because its absorbance maximum occurs at a wavelength different from that for norgestrel and norethindrone, i.e., 280 nm versus 240 nm.

Obviously, there is still a need for a rapid and automated clean-up, and determination of ethynyl steroids in urine at trace levels. We developed such a method by combining selective on-line trace enrichment using hydrophobic and Ag(I)-loaded sorbents, with liquid chromatography and dual-wavelength detection.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps - both equipped with home-made membrane pulse dampeners -, an MCS 670 Tracer valve switching unit and a Model 200 Programmer was used, in combination with two Kratos (Ramsey, NJ, USA) Model Spectroflow 757 UV-absorbance detectors. Chromatograms were either analog recorded at 240 and 280 nm using two Kipp & Zonen (Delft, The Netherlands) BD 40 recorders, or digital via an Anacomp (Kontron) Model 220 computer.

Stationary phases and columns

On-line trace enrichment and clean-up was performed on Chrompack (Middelburg, The Netherlands) 10 x 2.0 mm I.D. preconcentration columns packed by hand using a syringe containing a slurry of 10 µm styrene-divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, USA) or 25-40 µm Ag(I)-loaded Spheron Oxine 1000 (Lachema, Brno, Czechoslovakia) in methanol.

A 100 x 3 mm I.D. glass cartridge prepacked with 5 µm Chromspher (Chrompack) octadecyl-bonded silica and equipped with a 10 x 2 mm I.D. guard column packed with 5 µm LiChrosorb RP-18 served as analytical column.

Preparation of the Ag(I)-oxine phase

An appropriate amount of silver(I) acetate was dissolved in 10^{-3} M acetic acid in order to obtain a 0.05 M solution. The pH value thus obtained should be in the order of 6.2. 0.5 g of Spheron Oxine 1000 was added and the mixture was mechanically shaken

for two hours. The Ag(I)-loaded phase was collected on a glass filter and washed with 30 ml LC-grade water and 30 ml methanol, respectively, and dried at room temperature in the dark. The Spheron Oxine material could be regenerated by washing with acetonitrile-water (50:50), acidified with perchloric acid (pH 1.9) and, next, water and methanol. After drying at room temperature, the oxine phase is ready for re-loading with Ag(I).

Chemicals

Analytical-grade acetic acid, nitric acid, perchloric acid, ethanol, HPLC-grade methanol and HPLC-gradient grade acetonitrile were obtained from J.T. Baker (Deventer, The Netherlands). Silver acetate was obtained from Aldrich (Beerse, Belgium). Pharmaceutical-grade ethynylestradiol, norgestrel and norethindrone were obtained from Sigma (St. Louis, MO, U.S.A.); their structures are given in Fig. 1. A stock solution of the steroids ethynylestradiol (2.5 μg/ml), norgestrel (5 μg/ml) and norethindrone (5 μg/ml), was prepared in ethanol, stored at 4°C in the dark and diluted with LC-grade water or urine, prior to use. Urine samples were paper-filtered prior to the on-line trace enrichment.

LC-grade water was prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system. Eluents were degassed under vacuum in an ultrasonic bath. The pH was adjusted after the addition of acetonitrile using a Philips (Eindhoven, The Netherlands) PW 9409 pH meter.

RESULTS AND DISCUSSION

Characteristics of the precolumns

Breakthrough experiments were carried out according to the procedure described in ref. 8, using a 250 ng/ml standard solution of ethynylestradiol in water. At a flow-rate of 5 ml/min the breakthrough volume on the 10 x 2 mm I.D. PRP_1 precolumn was found to be more than 50 ml, which was the maximum volume tested.

The choice of the eluent for the desorption of the steroids from the PRP_1 precolumn and their subsequent sorption on the Ag(I)-oxine precolumn was taken from ref. 4. Methanol-water (70:30) provided an satisfactory desorption from the PRP_1 precolumn; in addition, the breakthrough volume for ethynylestradiol dissolved in this eluent on the 10 x 2 mm I.D. Ag(I)-oxine precolumn was found to be more than sufficient: at least 20 ml at a flow-rate of 0.5 ml/min. It should be noticed that retention on the Ag(I)-oxine is flow-rate dependent [5]; for safety reasons we used always 1.0 ml/min during this step of the solid phase extraction procedure.

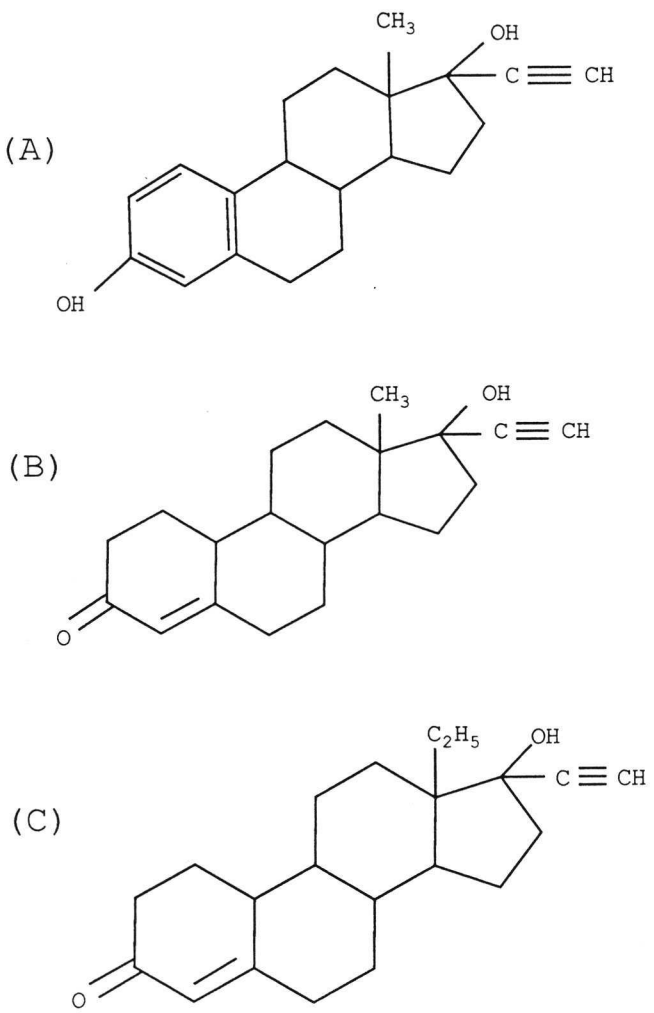

Fig. 1. Structures of ethynylsteroids under investigation. A, ethynylestradiol; B, norethindrone; C, norgestrel.

The mobile phase used for the desorption of the ethynyl steroids from the Ag(I)-oxine precolumn was found to be more critical. We used a relatively low acetonitrile content in order to be sure to obtain a peak compression effect (cf. below) on the top of the C18 analytical column. The peak shape improved considerably after a step gradient from 15 to 50% acetonitrile, which had been substituted for an isocratic elution at a 40% modifier content. The pH of the mobile phase used for desorption from the Ag(I)-oxine was very critical. At a pH of over 2.5, no desorption occurred at all. We used acetonitrile-water (15:85) acidified to pH 1.9, but still at least 2 ml of eluent were necessary in order to obtain satisfactory desorption. Probably the unfavourable elution profile is not due to the pH value but to the low acetonitrile content which is not able to provide an efficient elution because of the relatively strong reversed-phase nature of the bare Spheron Oxine. In practice, however, this drawback could be easily overcome by using backflush desorption and creating peak compression on top of the analytical column.

The choice of the acid for desorption was not critical; both nitric and perchloric acid yielded acceptable results. This indicates that the desorption from the Ag(I)-oxine is primarily a neutralization process. We preferred the use of perchloric acid because of its lower background at low detection wavelengths.

General procedure

Final experiments were performed using the set-up shown in Fig. 2. The procedure is as follows. The PRP_1 precolumn is wetted by 5 ml methanol and conditioned with 10 ml water via pump A at 5 ml/min. Then 20 ml of a standard steroid mixture or a urine sample are loaded onto the PRP_1 precolumn at 4 ml/min. Here sorption of the ethynyl steroids and of various organic contaminants occurs, while inorganic and water-soluble constituents, e.g., chloride ions and amino acids, are flushed to waste. The PRP_1 precolumn is flushed with 10 ml of water and 10 ml of methanol-water (50:50) in order to be sure that contaminants which might interfere with the sorption process on the second precolumn are flushed to waste. Next, the Ag(I)-oxine precolumn is conditioned with 2 ml of methanol-water (70:30). The steroids are eluted with another 4 ml (in the forward-flush mode, at 1.0 ml/min) from the PRP_1 precolumn to the Ag(I)-oxine precolumn on which they are selectively trapped in a narrow zone by complexation, while organic interferences, e.g., endogeneous steroids not having the ethynyl group, are flushed to waste. Then the Ag(I)-oxine precolumn is flushed with 2 ml of methanol-water (70:30) and, next, 2 ml of water at 2 ml/min; the latter in order to remove all methanol prior to the next (elution) step. Finally, the ethynylsteroids are desorbed in the backflush

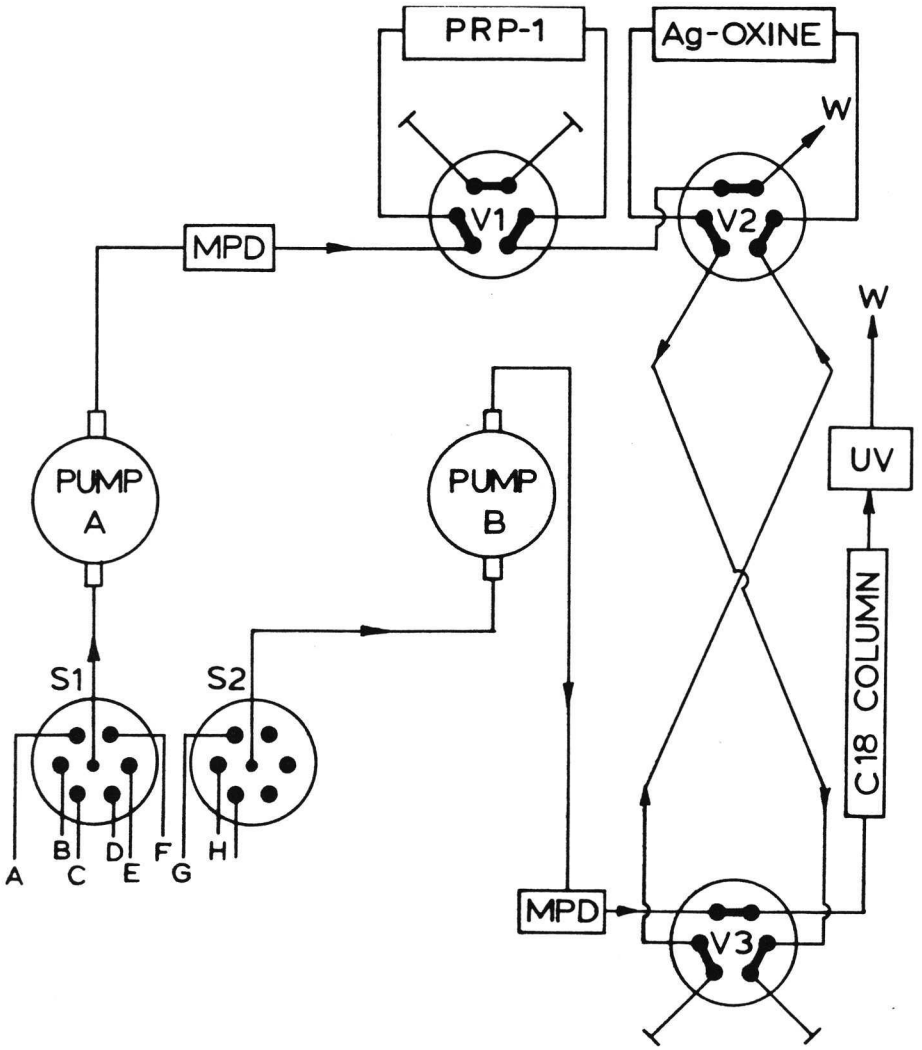

Fig. 2. Experimental set-up for the on-line trace enrichment and determination of ethynyl steroids in urine.

S_1 and S_2, low-pressure solvent selection valves; V_1, V_2 and V_3, high-pressure switching valves; MPD, membrane pulse dampener; W, waste; A, water; B, methanol; C, water; D, sample; E, methanol-water (50:50); F, methanol-water (70:30); G, acetonitrile-water (15:85) acidified with perchloric acid (pH 1.9); H, acetonitrile-water (50:50). Precolumns: 10 x 2.0 mm I.D. PRP_1 and Ag(I)-oxine. Analytical column: 100 x 3.0 mm I.D. C18, equipped with a 10 x 2.0 mm I.D. guard column. Flow-rate of pump A, 0.5-5 ml/min; flow-rate of pump B, 0.6 ml/min.

mode by 2.4 ml of acidified (pH 1.9) acetonitrile-water (15:85) via pump B and transferred to the C18 analytical column (which has been conditioned with the same mobile phase), on top of which peak compression occurs. The separation is achieved by a step gradient to acetonitrile-water (50:50) and the hormones are detected by UV absorbance at 240 and 280 nm. In the present study, the Ag(I)-oxine precolumn were replaced by hand prior to the next run. However, complete automation can easily be obtained by using a microprocessor controlled cartridge exchange device, as recently described by Nielen et al. [9].

In order to increase sample throughput, the next run is started while the actual separation is still in progress. Consequently, an analysis takes 30 min only. The general procedure has been summarized in Table I; details concerning the valve-switching program will be made available on request.

Table I. General procedure using the set-up of Fig. 2.

1.	Trapping of the ethynylsteroids on PRP_1.
2.	Flushing of PRP_1 with water and methanol-water (50:50).
3.	Transfer of steroids to Ag(I)-oxine with methanol-water (70:30).
4.	Flushing of Ag(I)-oxine with methanol-water (70:30) and with water.
5.	Conditioning of C18 analytical column with acidified acetonitrile-water (15:85).
6.	Backflush desorption from Ag(I)-oxine to the C18 analytical column using acidified acetonitrile-water (15:85).
7.	Step gradient to acetonitrile-water (50:50).

Performance and application

With the experimental set-up shown in Fig. 2 and the general procedure described in Table I, analytical data were collected for ethynylestradiol, norgestrel and norethindrone. The results which are presented in Table II, clearly demonstrate the potential of the method.

Table II. General performance of the automated analysis of ethynylsteroids according to the procedure in Table I and using the set-up of Fig. 2.

20-ml samples spiked at the 5-10 ng/ml level.

Compound	Repeatability (% rel. S.D.)		Recovery (%)		Detection limit
	standard	urine	standard vs. loop (peak area)	urine vs. standard (peak height)	(ng/ml urine; S:N = 3:1)
n =	10	10	3	3	3
Norethindrone	2.5	2.2	72	86	0.10
Ethynylestradiol	2.7	3.5	67	92	0.25
Norgestrel	3.4	3.4	76	102	0.12

The recovery for the complete sorption/desorption procedure was determined by comparing the peak areas obtained with a direct 113-µl loop injection onto the C18 analytical column and a 20-ml on-line trace enrichment of the same (but proportionally diluted) sample, using the system of Fig. 2 and the procedure in Table I. The recovery for standard solutions was found to be satisfactory but not quantitative. The losses are probably due to a non-complete desorption from the Ag(I)-oxine precolumn. In spite of this the repeatibility was very good; therefore no internal standard was required.

The recovery from spiked urine as compared to a standard solution was between 86 and 102%. The repeatability for spiked urine samples was between 2.2 and 3.5%. Detection limits in urine samples are at the sub-ng per ml level.

Figs. 3 and 4 show chromatograms obtained with the diode array detector set at 240 and 280 nm, respectively. One should note that, in the latter case, a 10-fold increased detector sensitivity has to be used because of the lower molar extinction coefficient of ethynylestradiol. Figs. 3a and 4a show 20-ml standard samples spiked with 10 ng/ml norethindrone and norgestrel, and 5 ng/ml ethynylestradiol. The selectivity obtained by the use of two different wavelengths is demonstrated by the absence of ethynylestradiol at 240 nm and the small norethindrone and norgestrel peaks at 280 nm. Figs. 3b and 4b show 20-ml urine samples spiked at the same level as the standard. The excellent recovery as compared to the standard solution is obvious and the good selectivity allows the identification of these hormones by their UV characteristics in combination with the

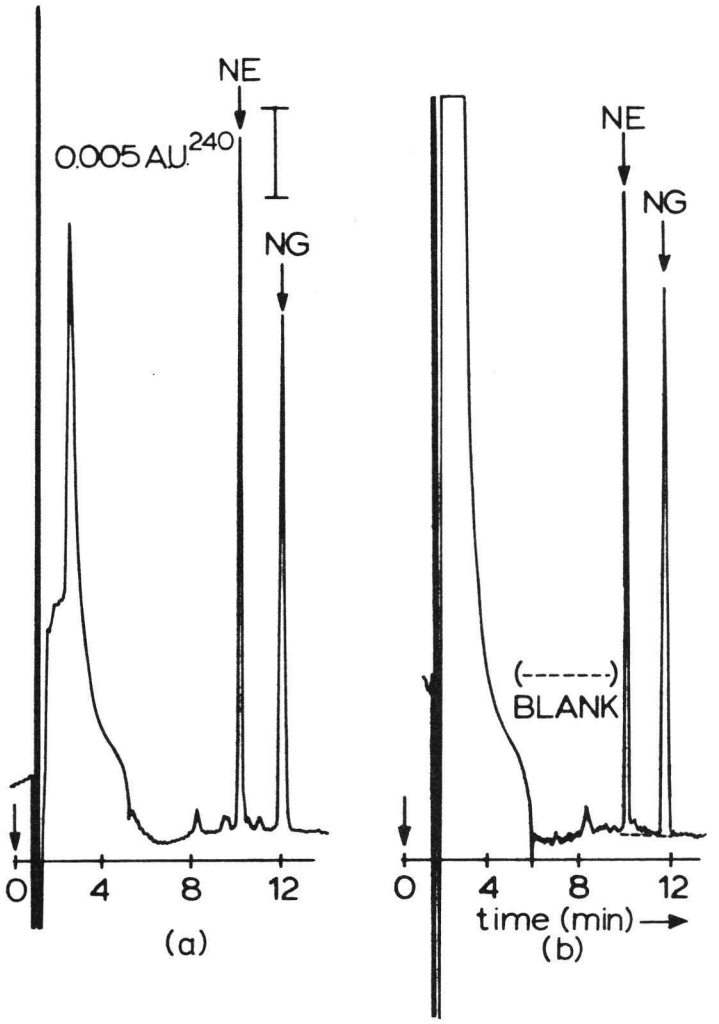

Fig. 3. Chromatograms of 20 ml samples spiked with 10 ng/ml norethindrone (NE), norgestrel (NG) and 5 ng/ml ethynylestradiol (EE), and analyzed according to the procedure in Table I using the set-up of Fig. 2. Detection at 240 nm. Samples: (a) standard; (b) urine.

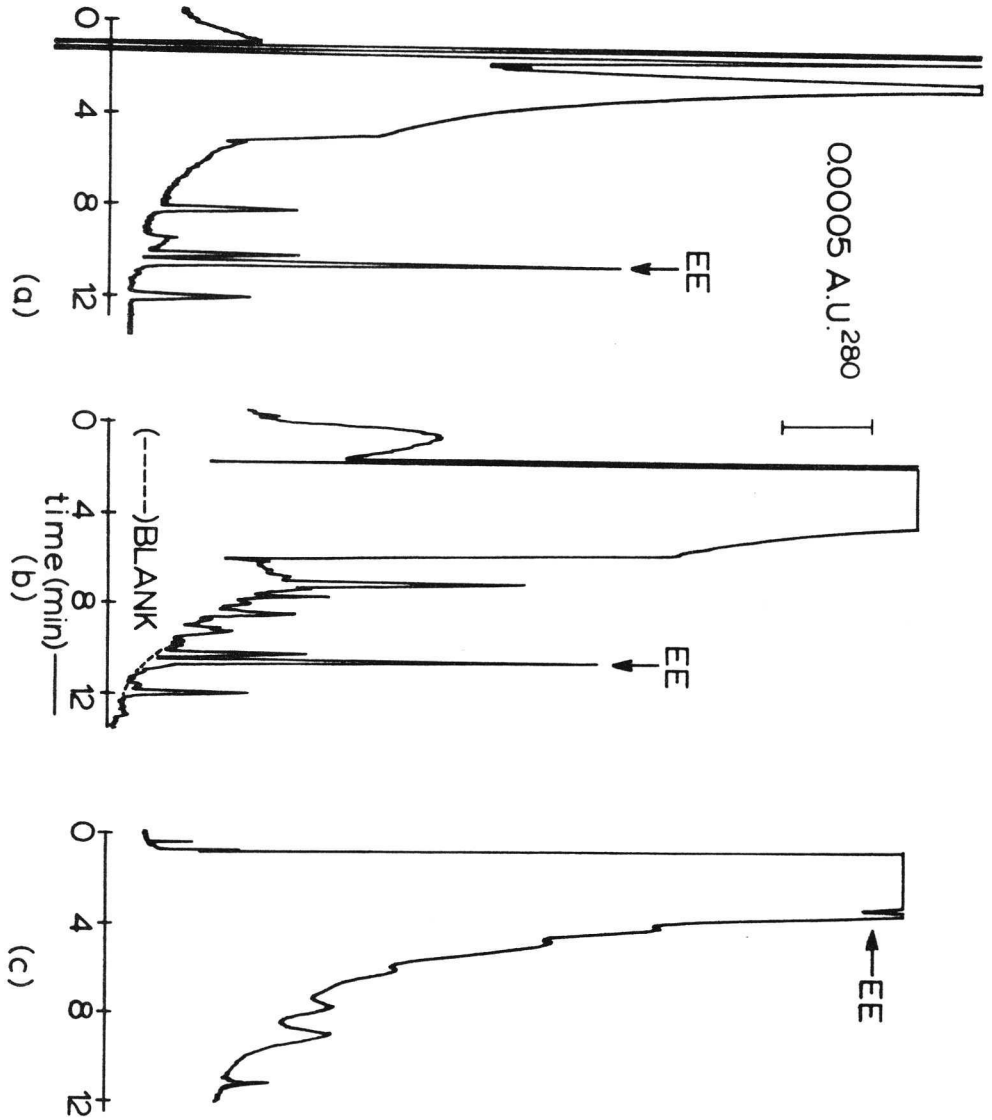

Fig. 4. Analyses of the same samples as in Fig. 3, except for the detection wavelength (280 nm) and the 10-fold higher detector sensitivity. Sample (c), urine but using the PRP$_1$ precolumn only.

retention times.

Fig. 4c shows a chromatogram for the same urine sample as analyzed before but using the PRP_1 precolumn only. After flushing the precolumn with 10 ml of water and methanol-water (50:50), the steroids were desorbed isocratically by acetonitrile-water (40:60) to the C18 analytical column and detected at 280 nm. The benefit of selective trace enrichment using the dual-precolumn approach is clearly evident for the determination of ethynylestradiol in real samples.

CONCLUSIONS

Using two precolumns in series, with cleaning action being based on the removal of, first, inorganic and other water-soluble and, then, organic compounds, urine samples can be analyzed for ethynyl steroids with good sensitivity and selectivity; even when using UV detection at low wavelengths. Additional selectivity is obtained by recording two different detection wavelengths simultaneously. The present automated method allows the rapid screening of urine samples for ethynyl steroids without time-consuming clean-up and derivatization, and without the use of expensive GC-MS equipment [3,4].

The remaining off-line manipulations - i.e., the replacing of the precolumns and the hydrolysis of conjugates (necessary in case of patient samples) - can be also automated by using a microprocessor controlled cartridge exchanger [9] and a column packed with immobilized glucuronidase [10,11], respectively. The application of immobilized enzymes to the automated de-glucuronidation of large volumes of urine is currently under investigation.

Acknowledgements

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References

1. Martindale, "The Extra Pharmacopoeia", 27th ed., The Pharmaceutical Press, London, 1979.
2. R. Heikkinen, Th. Fotsis and H. Adlercreutz, J. Steroid Biochem., 19 (1983) 175.
3. M. Tetsuo, M. Axelson and J. Sjövall, J. Steroid Biochem., 13 (1980) 847.
4. S.H.G. Andersson, M. Axelson, B.-L. Sahlberg and J. Sjövall, Anal. Lett., 14 (1981) 783.
5. M.W.F. Nielen, H.E. van Ingen, A.J. Valk, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., in press (1986).
6. J. de Jong, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 381 (1986) 431.
7. A.N. Papas, S.M. Marchese and M.F. Delaney, LC Magazine 3 (1985) 354.
8. C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 53 (1981) 2072.
9. M.W.F. Nielen, A.J. Valk, R.W. Frei, U.A.Th. Brinkman, Ph. Mussche, R. de Nijs, B. Ooms and W. Smink, J. Chromatogr., submitted for publication (1986).
10. L.D. Bowers and P.R. Johnson, Anal. Biochem., 116 (1981) 111.
11. A. Farjam, personal communication.

Chapter 5. Miniaturization of precolumn technology

5.1 General considerations

During the past few years, increasing interest has been shown in the use of narrow-bore separation columns (< 2 mm I.D.) in liquid chromatography (LC), as demonstrated by the growing number of reviews and books in this field[1-5]. Advantages are reduced cost (less packing material, reduced solvent consumption), high mass sensitivity and more convenient interfacing with, e.g., gas chromatographic detectors, mass spectrometers and Fourier transformed infra-red spectrometers.

Apart from the instrumental complications caused by the requirement that additional band broadening should be extremely small, the major disadvantage of miniaturized LC is the reduced concentration sensitivity due to the limited injection volume of about 1 µl (1 mm I.D. columns) and the diminished pathlength in UV absorption detector flow-cells. Poppe and Kraak[6] concluded that narrow-bore LC often is not the correct choice when carrying out trace analyses. To solve this problem, several authors include trace enrichment prior to the actual analysis. Ishii et al.[7] used a micro-precolumn which is loaded off-line and then placed on the top of the separation column; automation is impossible. Krejci[8] and Apffel et al.[9] used on-column preconcentration applying the peak-compression principle, but column lifetime was reduced considerably when real samples were analyzed. Kok et al.[10] designed a micro-precolumn for an on-line system which performed quite well; the main disadvantage was the impossibility of a continuous flow through the separation column. A relatively long narrow-bore precolumn was used by Scott and Kucera[11] in combination with gradient elution which seems to be essential there for good overall performance. Obviously, despite all these attempts, there still is a need for a simple micro-precolumn which can be packed manually and used in on-line (automated) systems.

In biomedical analysis, the available sample volume is much higher than the usual injection volume of 1 µl and generally is in the 100-1000 µl range. This means that narrow-bore LC with on-line trace enrichment is especially suitable for biomedical applications. Enrichment factors of about 100 should be obtainable when 100 µl samples are used and desorption is efficient. In recent years, direct injection of biomedical samples on a conventional-size precolumn has become increasingly popular[12-18]. In several studies, relatively large precolumns (30 x 4.6 mm I.D.) were used which typically contain 30-µm C-18 particles. Various applications with (semi-) automated systems have appeared in the literature. For example, Juergens[17] used short (5 x 4.6 mm I.D.)

precolumns for the direct injection of serum samples. Because of the short length, backflush desorption (usually applied with long precolumns in order to prevent excessive additional band broadening) was not necessary any more and the forward desorption used reduced the precolumn clogging problems often caused by serum samples.

In our group, various types of precolumns have been designed for conventional-size systems (4.6 mm I.D.) and they have been routinely applied in several research projects[19]. Recently, we developed a 2 mm I.D. precolumn based on the same design and used it with 3 mm I.D. LC systems[20]; today, it is also coupled to 2 mm I.D. separation columns for on-line LC-mass spectrometry[21]. However, when further decreasing the (pre)column diameter, i.e., to 1 mm I.D., several problems arise. From a construction point of view, further reduction of the previous design[19] is almost impossible and, besides, the design does not meet the requirements of narrow-bore LC such as, especially, negligible band broadening. In other words, one has to develop a completely different type of precolumn for narrow-bore work. The following considerations have to be taken into account.

A micro-precolumn should be used efficiently, because of the small amount of packing material present. That is, there should be a flow-spreading device on the top of the precolumn, which effects spreading of the sample over the total diameter of the precolumn. Usually, the top of the precolumn is sealed with a porous frit, but with direct plasma injections metal sieves (screens) are preferred to prevent clogging of the precolumn by protein fragments[16]. The use of screens instead of porous frits also reduces band broadening. However, screens will give no flow spreading which means that the inner diameter of the inlet capillary and the flow-rate during the loading step may play an important role with regard to the efficiency of the precolumn and the reproducibility of its performance. In preliminary experiments[22] it was found that reproducible breakthrough volumes can be obtained when using 1 mm I.D. micro-precolumns with inner lengths of 4-5 mm, in combination with inlet capillaries having inner diameters of over 0.5 mm, and provided with screens. In addition, the flow-rate during the loading step should be preferably less than 500 µl/min.

Direct plasma injection without any clean-up requires wide-bore (> 0.5 mm I.D.) connecting capillaries[16]. However, in our preliminary study this was found to be hardly compatible with the requirements of high-efficiency narrow-bore LC where band broadening should be as low as possible. Consequently, in the design of an automated precolumn system for trace enrichment and clean-up of plasma samples for narrow-bore LC, special attention should be paid to the possibility of direct plasma injection without excessive additional band broadening.

References

1. R.P.W. Scott, J. Chromatogr. Sci., 18 (1980) 49.
2. M. Novotny, Anal. Chem., 53 (1981) 1294A.
3. F.J. Yang, J. High Res. Chromatogr. & Chrom. Comm., 6 (1983) 348.
4. P. Kucera ed., Microcolumn High-Performance Liquid Chromatography. Elsevier Scientific Publishing Company, Amsterdam (1984).
5. R.P.W. Scott ed., Small Bore Liquid Chromatography Columns: Their Properties and Uses, John Wiley & Sons, New York (1984).
6. H. Poppe and J.C. Kraak, J. Chromatogr., 255 (1983) 395.
7. D. Ishii, K. Hibi, K. Asai and M. Nagaya, J. Chromatogr., 152 (1978) 341.
8. M. Krejci, presented at the Workshop on Low Dispersion Liquid Chromatography, Amsterdam, 1983.
9. J.A. Apffel, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 18 (1984) 5.
10. W.Th. Kok, U.A.Th. Brinkman, R.W. Frei and H.B. Hanekamp, J. Chromatogr., 237 (1982) 357.
11. R.P.W. Scott and P. Kucera, J. Chromatogr., 185 (1979) 27.
12. W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, J. Chromatogr., 222 (1981) 13.
13. K.G. Wahlund and T. Arvidson, J. Chromatogr., 282 (1983) 527.
14. W. Voelter, Th. Kronbach, K. Zech and R. Huber, J. Chromatogr., 239 (1982) 475.
15. A. Nazareth, L. Jaramillo, B.L. Karger, R.W. Giese and L.R. Snyder, J. Chromatogr., 309 (1984) 357.
16. W. Roth, J. Chromatogr., 279 (1983) 347.
17. U. Juergens, J. Chromatogr., 310 (1984) 97.
18. K. Zech and R. Huber, J. Chromatogr., 353 (1986) 351.
19. C.E. Goewie, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 301 (1984) 325.
20. M.W.F. Nielen, G. Koomen, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., 8 (1985) 315.
21. F.A. Maris, R.B. Geerdink, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 323 (1985) 113.
22. M.W.F. Nielen, E. Sol, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., 8 (1985) 1053.

5.2 *Automated precolumn system for trace enrichment and clean-up of plasma samples in narrow-bore liquid chromatography*

SUMMARY

The design of a switching valve having an internal pre-column for automated sample handling and trace enrichment in narrow-bore liquid chromatography is described. The system combines low dispersion with the capability of direct plasma injection without any (off-line) clean-up procedure. Band broadening and enrichment factors have been studied for 100-μl trace enrichment *versus* 0.5-μl direct loop injection. As an example, the automated analysis of plasma samples containing clobazam and desmethylclobazam at the low-ppb level is reported.

INTRODUCTION

Narrow-bore liquid chromatography (LC), featuring separation columns of about 1 mm I.D., offers advantages over conventional LC, such as relatively low consumption of solvent and stationary phase and high mass sensitivity. However, owing to the limited injection volume of about 1 μl and the reduced path length in, *e.g.*, UV absorption detector flow cells, concentration sensitivity is often unsatisfactory.

Recently, we have developed a simple micro-pre-column that can be packed manually and used in on-line (automated) systems[1]. Critical parameters such as pre-column length, inlet capillary I.D. and the use of screens instead of frits were studied and the applicability to the direct analysis of plasma and serum samples, *i.e.*, without any off-line clean-up, was demonstrated. Unfortunately, extra-column band broadening caused by this pre-column was serious and resulted in a 3.5-fold increase in detection limit and a 12-fold decrease in plate number. Therefore, the actual enrichment factor was 60 instead of the expected 200 for a 100-μl trace enrichment experiment *versus* a 0.5-μl loop injection.

In this paper we describe a micro-pre-column inserted within the axis of a common six-port switching valve. In this way, extra-column band broadening could be reduced without losing the benefits of the previous design. The system has been applied to the determination of clobazam and its active metabolite desmethylclobazam in plasma samples without any off-line sample pre-treatment.

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EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) Model 410 pump was used in combination with a Gilson (Villers le Bel, France) Model 302 pump and two modified Valco (Houston, TX, U.S.A.) six-port valves. For band broadening studies a home-made 0.5-μl injection valve modified from an early Jasco valve[2] was used. Valve switching was microprocessor-controlled using a Kipp & Zonen (Delft, The Netherlands) Model 5140 programmer. A Knauer (Bad Homburg, F.R.G.) fixed-wavelength (254 nm) photometer, equipped with a 1-μl flow-cell, was used for detection. Chromatograms were recorded on a Kipp & Zonen BD 40 recorder.

Chemicals

LC-grade water was obtained by purifying distilled water in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system. HPLC-grade acetonitrile was obtained from Promochem (Wesel, F.R.G.). Acetic acid was of analytical-reagent grade from Baker (Deventer, The Netherlands). The test mixture contained 3,5-dichlorophenol, 3,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol, which were purchased from Aldrich Europe (Beerse, Belgium).

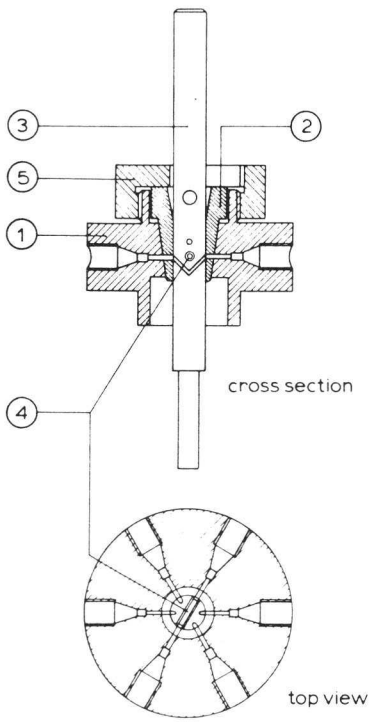

Fig. 1. Switching valve with internal micro-pre-column. For explanation, see text.

Stationary phases and columns

The analytical column was a 20 cm × 1 mm I.D. glass-lined stainless-steel column home-packed with 3-μm Spherisorb ODS-2 (Phase Separations, Queensferry, U.K.). The pre-column was packed with 40-μm Octyl (C_8) (Baker). Screens (36 μm) were obtained from Dinxperlo (Dinxperlo, The Netherlands).

Design of the switching valve with internal pre-column

The switching valve with its internal pre-column (Fig. 1) consists of three main parts, as follows.

(a) The valve body (1) is constructed from a standard Valco six-port switching valve. All ports were drilled to 1 mm. The analytical column fits directly into one of these ports without any connective tubing in between.

(b) The seal (2) is home-made and fits in the valve body. Note that this seal is fixed, whereas the seal in the standard switching valve is not.

(c) The axis of the valve (3) was drilled through, to give a 4.5 × 1 mm I.D. hole that can be filled with packing material to obtain the actual micro-pre-column (4). The axis contains one permanent screen; the other screen is held by a PTFE ring. The axis also contains two dummy channels for the other flow-lines of the six-port valve. These channels are drilled in such a way as not to touch each other, nor the micro-pre-column. The valve is leak-tight up to 250 bar when the wheel (5) has been tightened. The valve rotates extremely easily, thanks to the very small contact area between the rotating and the fixed part, and is easily pneumatically activated.

The geometrical volume between pre-column and separation column is about 2.5 μl, which is relatively high for narrow-bore LC. However, because of the absence of changes in inner diameter (all parts are 1 mm I.D.) the final band broadening caused by this dead volume is extremely small.

Pre-column packing procedure

For packing or emptying the pre-column, the axis is taken out of the switching valve and placed in a syringe adapter (Fig. 2). A syringe filled with a thin slurry of the packing material in methanol is fitted into the adapter and the pre-column is packed manually. Excess of packing material at the pre-column inlet is removed after the axis has been taken out again, and a screen and PTFE ring are inserted to retain the packing material. Then the axis is placed in the switching valve again and the wheel is tightened; the pre-column is now ready for use. The precolumn is emptied in a similar way by using a syringe filled with methanol, fitting the syringe into the adapter from the opposite side.

The entire packing procedure takes less than 5 min. It was not necessary to repack the pre-column frequently because of the routinely used on-line regeneration. Arbitrarily, we repacked the pre-column once a week.

RESULTS AND DISCUSSION

Band broadening due to the micro-pre-column/switching valve

In order to determine the contribution of the micro-pre-column/switching valve to the total band broadening of the LC system, a comparison was made between 0.5-μl loop injections of the chlorophenol test mixture (1 mg/ml dissolved in the

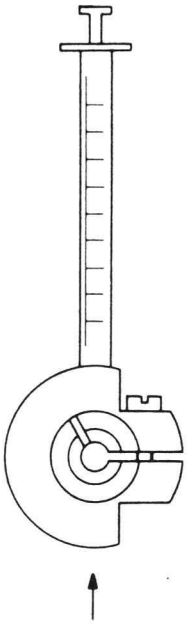

Fig. 2. Syringe adapter for manual packing and emptying (arrow position) of the micro-pre-column.

mobile phase) and 100-μl trace enrichment on the micro-pre-column after 200-fold dilution of the test mixture with 0.05% acetic acid.

Desorption after trace enrichment was carried out in both the forward- and the back-flush mode and all experiments were carried out in triplicate. From the results given in Table I, it can be seen that there is only a 30% (1.3-fold) increase in detection limit and a 40% (1.7-fold) decrease in plate number caused by extra-column

TABLE I

INFLUENCE OF MICRO-PRE-COLUMN BAND BROADENING ON SYSTEM EFFICIENCY AND DETECTION LIMITS

Conditions: 20 cm × 1 mm I.D. 3-μm Spherisorb ODS-2 column, acetonitrile–0.11% acetic acid (55:45) at 50 μl/min; N = 10 000 for test mixture; 100-μl (5 μg/ml) trace enrichment (flush volume 800 μl of 0.05% acetic acid) *vs.* 0.5-μl loop injection (1 mg/ml).

Compound	*k'*	*Increase in detection limit*		*Decrease in plate number*	
		*FF**	*BF**	*FF*	*BF*
3,5-Dichlorophenol	3	1.3	1.2	1.7	1.4
2,4,5-Trichlorophenol	4	1.3	1.3	1.7	1.7
2,3,4,6-Tetrachlorophenol	6	1.3	1.2	1.7	1.4

* FF, forward-flush desorption; BF, back-flush desorption.

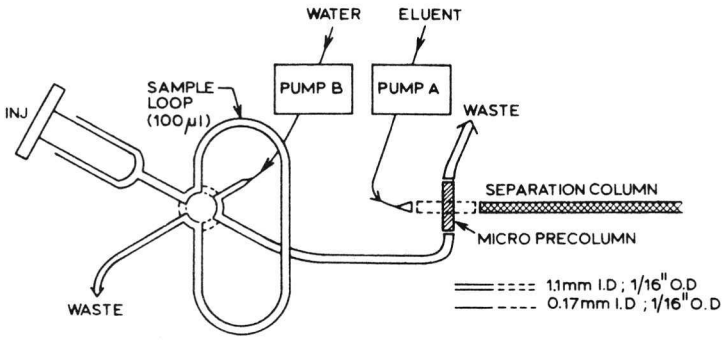

Fig. 3. Set-up for the automated sample handling and trace enrichment of plasma samples. Conditions: 20 cm × 1 mm I.D. 3-µm Spherisorb ODS-2 column, acetonitrile–water (50:50) at 50 µl/min; detection at 254 nm, 0.02 a.u.f.s. Micro-pre-column, 4.5 × 1 mm I.D. 40-µm Octyl (C_8). Sampling at 200 µl/min.

band broadening. Hence, the actual increase in sensitivity as a result of trace enrichment will be 150 rather than the expected value of 200.

One should note the small difference between back-flush and forward-flush desorption. This difference indicates that retention on the pre-column during the elution step is not negligible; that is, the extra-column band broadening must be attributed to the kind of packing material rather than to the design itself. When untreated plasma samples have to be analysed, we prefer the forward-flush mode in order to maintain the protective filter aspect of the pre-column; in this way no blocking of the analytical column will occur.

Automated handling of plasma samples

As an example, the micro-pre-column was used for the automated handling of untreated plasma samples spiked with the tranquillizer clobazam and its active metabolite desmethylclobazam; the set-up used is shown in Fig. 3.

After filling the loop, the switching programme summarized in Table II was

TABLE II

SWITCHING PROGRAMME FOR THE AUTOMATED ANALYSIS OF CLOBAZAM AND DESMETHYLCLOBAZAM IN PLASMA, USING THE SET-UP IN FIG. 3

Conditions: 20 cm × 1 mm I.D. 3-µm Spherisorb ODS-2 column, acetonitrile–water (50:50) at 50 µl/min; detection at 254 nm, 0.02 a.u.f.s. Micro-pre-column, 4.5 × 1 mm I.D. 40-µm Octyl (C_8). Sampling at 200 µl/min.

Time (sec)	*Event*
0	Sample loop (100 µl) filled
1	Inject on to micro-pre-column and flush with 3000 µl of water
900	Forward-flush desorption with 50 µl of eluent
960	Reset pre-column valve; flush pre-column with 1800 µl of water
1497	Reset sampling valve
1500	End

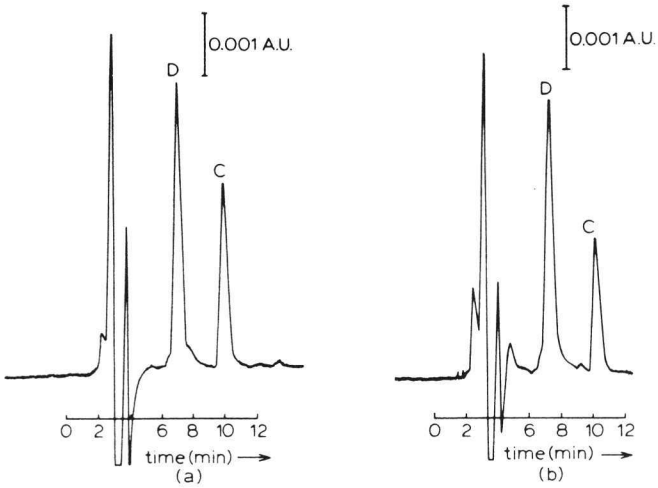

Fig. 4. Chromatograms of a 100 ng/ml standard solution of desmethylclobazam (D) and clobazam (C) diluted 1:1 with water (a) and with blank plasma (b). Conditions as in Table II and Fig. 3.

started. A 100 ng/ml standard solution of clobazam and desmethylclobazam in water was diluted with an equal volume of water or with fresh human plasma and concentrated on the micro-pre-column and analysed (Fig. 4a and b). During each analytical separation the pre-column was flushed on-line with, arbitrarily, about 2 ml of water to remove the remaining water-soluble protein fragments and then re-used for the next sample. From the chromatogram it can be seen that the sorption–desorption procedure on the pre-column provides excellent clean-up; even with the non-selective UV detection at 254 nm, no interferences are observed.

Relevant analytical data are given in Table III. The detection limits are 2.5 and 5 ng/ml in real samples for desmethylclobazam and clobazam, respectively, and are comparable to those in conventional LC with post-column photochemical reaction and fluorescence detection[3,4].

TABLE III

ANALYTICAL DATA FOR THE AUTOMATED ANALYSIS OF CLOBAZAM AND DESMETHYLCLOBAZAM IN FRESH PLASMA

Conditions: 100 µl samples, analysed following the switching programme in Table II.

Compound	*Repeatability (%) for*		*Recovery (%) from plasma*	*Detection limit in plasma samples*	
	Standards (n = 10)	*Plasma (n = 6)*	*(n = 6)*	*ng*	*ng/ml*
Desmethylclobazam	±0.7	±1.7	91	0.25	2.5
Clobazam	±2.2	±1.1	71	0.5	5

CONCLUSION

The sample capacity in narrow-bore LC can be successfully improved by on-line trace enrichment on a micro-pre-column. An increase in sensitivity of more than two orders of magnitude can easily be obtained (100-μl trace enrichment *versus* 0.5-μl loop injection). The present switching valve with its internal pre-column allows automated sample handling and trace enrichment without excessive additional band broadening ($\sigma_{v,\text{pre-column}}$ = 3.5 μl; k' = 3). Even with a high-performance analytical system (N = 10 000), 60% of the separation power is maintained, which contrasts favourably with earlier results[1] where over 90% of the separation power was lost. The additional band broadening apparently is mainly caused by the nature of the packing material in the pre- and analytical columns and not by the pre-column design; in other words, a further reduction in band broadening should be attainable.

The present design, which features wide-pore screens, relatively large packing material (d_p 40 μm) and wide-bore valve ports, allows the repeated injection of fresh plasma samples without clogging of the system, as has been demonstrated for clobazam and desmethylclobazam with good repeatability and detection limits at the low ng/ml level.

The on-line combination of several pre-columns packed with different types of packing materials for an increase in selectivity in the handling of more complex samples, using automated narrow-bore LC systems, is currently under investigation.

ACKNOWLEDGEMENTS

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REFERENCES

1 M. W. F. Nielen, E. Sol, R. W. Frei and U. A. Th. Brinkman, *J. Liq. Chromatogr.*, (1985) in press.
2 T. Takeuchi and D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 469.
3 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta*, 114 (1980) 137.
4 M. Uihlein and E. Schwab, *Chromatographia*, 15 (1982) 140.

Chapter 6. A fully automated sample handling system for liquid chromatography based on precolumn technology and automated cartridge exchange

SUMMARY

The design of an automated cartridge exchange module for on-line sample handling in liquid chromatography is described. When combined with a low-cost purge pump, a solvent selection valve and an auto-sampler, a fully automated sample handling system is obtained. Samples are sorbed on a disposable cartridge packed with 40 μm octyl-bonded silica, purged for clean-up and eluted on-line to the analytical column. Unattended operation of the system is demonstrated for various examples, i.e., the determination of anti-epileptic drugs in serum, an anti-cancer drug in plasma, barbiturates in urine, phenylurea herbicides in river water, and caffeine in a soft drink.

INTRODUCTION

Nowadays, sample handling is often the time-determining step in liquid chromatography (LC). Despite the use of automated LC systems, and auto-samplers, for many LC analyses sample preparation is almost invariably done manually. However, on-line coupling of sample handling steps to a liquid chromatograph via precolumn technology is very attractive[1]. In this case, basically solid phase extraction procedures are employed, i.e., the sample is loaded on a suitable sorbent, purged for clean-up and finally eluted on-line to the analytical column.

Recently, McDowall et al.[2] evaluated liquid-solid sample preparation in drug analysis. The advantages of solid phase extraction as compared to traditional (manual) liquid-liquid extraction procedures were pointed out and an overview was given of the state-of-the-art of manual and automated procedures. In addition, off-line and on-line (referred to in their paper as 'column-switching') procedures were compared. The authors observe that the development of a new generation of fully automated sample preparation systems is essential to exploit fully the advantages of solid phase extraction.

The impact of sample handling via solid phase extraction procedures has been clearly recognized by several companies. Table I summarizes some different approaches which are commercially available. Except for the (pre)column switching methods and the automated sample handling (PROSPEKT) system described in this paper, none of the other systems is fully automated. For the PROSPEKT approach, we designed a low-cost disposable cartridge and a simple cartridge transport system, which can be controlled by,

Table I. Automation Potential of Commercially Available solid phase Extraction Systems.

Event	Baker-10[3]	Bond-Elut[4]	Du Pont Prep[5]*	AASP[6]	PROSPEKT**	(Pre)column switching[1,7]
Sampling	off-line	off-line	on-line	off-line	on-line	on-line
Purging	off-line	off-line	on-line	on-line	on-line	on-line
Elution	off-line	off-line	off-line	on-line	on-line	on-line

*Not available anymore

**Present paper

e.g., the built-in microprocessor of an auto-sampler.

General considerations

Several authors describe the routine use of on-line precolumns for automated solid phase extraction prior to LC[7-12]. A trend towards the use of relatively small precolumns (length, 2-10 mm; I.D., 2-4.6 mm) packed with rather large (d_p between 25 and 60 μm) particles; equipped with stainless-steel sieves instead of porous frits and connected with relatively wide-bore (0.5 mm I.D.) capillaries can be clearly recognized. Obviously, preventing the blocking of the sytem by protein fragments has been an important aspect in the design of these precolumns which, in most cases, are used for a number of subsequent analyses. Most workers apply 5-250 μl of serum or plasma samples to such precolumns, but usually after initial dilution, filtration, centrifugation and/or pH adjustment, which is necessary for the proper functioning of the system. Depending on the clean-up achieved via these off-line manipulations, up to a total of 20 ml plasma or serum can be applied, independently of the individual injection volumes, before the precolumn which is cleaned after each analysis and the guard column have to be replaced.

On the other hand, if one intends to take full advantage of the automation potential, off-line manipulations should be reduced to the absolute minimum. This unavoidably will result in a serious reduction of the precolumn lifetime. In addition, when the concentration of the analyte(s) varies over a wide range, the precolumn should be exchanged frequently to prevent memory effects. In essence, from the point of view of

quality assurance, it is preferable to use a fresh precolumn for each single analysis. Finally, there is another important aspect, i.e., the use of more selective precolumn sorbents, such as ion-exchangers[13,14] and metal-loaded phases[15]. Regeneration of these precolumn materials is often tedious, time-consuming and sometimes even impossible.

For the above reasons there obviously is a need for a sample handling system combining the advantages of precolumn/cartridge exchange (with its inherent quality assurance) and the automation aspects of precolumn technology. In this paper we describe such a system which consists of a microprocessor-controlled auto-sampler, a simple cartridge exchanger, a low-cost purge pump and a low-pressure solvent selection valve. The retention on the catridges, extra-column band broadening, repeatability and analyte recovery were studied and the feasibility of the approach was demonstrated for five different matrices, i.e., serum, plasma, urine, river water and a soft drink.

EXPERIMENTAL

Apparatus

A Kipp & Zonen (Delft, The Netherlands) Model 4140 pump, equipped with a Kontron (Zürich, Switzerland) pulse dampener was used in combination with a Kratos (Ramsey, NJ., U.S.A.) Spectroflow 757 variable-wavelength UV absorbance detector. The automated sample handling system consisted of a Spark Holland (Emmen, The Netherlands) Model Promis auto-sampler and a prototype of a Spark Holland PROSPEKT system, consisting of a cartridge exchange module, a low-cost purge pump (LDC-Milton Roy minipump VS, Riviera Beach, FL, U.S.A.) and an electrically operated low-pressure six-port solvent selection valve (Latek TMV, Heidelberg, F.R.G.). Chromatograms were recorded and processed using an Anacomp (Kontron) Model 220 computer. The sample handling system and the computer were controlled and started by the auxillary contact closures of the Promis auto-sampler.

For comparison purposes, some experiments were performed with the Advanced Automated Sample Processor (AASP; Varian, Sunnyvale, CA, U.S.A.) which was loaded with 20 x 2 mm I.D. 40 µm octyl-bonded silica cartridges from Analytichem (Harbor City, CA, U.S.A.).

Stationary phases and columns

The analytical column was a 10 or 20 cm x 3.0 mm I.D. ChromSep (Chrompack, Middelburg, The Netherlands) 5 µm octadecyl-bonded silica column, equipped with a 10 x 2 mm I.D. guard column, prepacked with pellicular C18 material. The

precolumns/cartridges were manufactured by Chrompack on our request; they are shown in Fig. 1. These cartridges (length, 10 mm; I.D., 2 mm; O.D., 10 mm) which are constructed from PVDF, are pressure-resistant up to at least 400 bar. They were slurry-packed with 40 μm octyl-bonded silica (Baker, Deventer, The Netherlands) and sealed by two 25 μm stainless-steel sieves (Dinxperlo B.V., Dinxperlo, The Netherlands).

Chemicals

LC-grade methanol, LC-gradient grade acetonitrile and analytical-grade phosphoric acid, potassium dihydrogen phosphate and acetic acid were obtained from Baker. 3,5-Dichlorophenol and caffeine were obtained from Aldrich (Beerse, Belgium). The anti-epileptic drugs primidone, phenobarbital, phenytoin and carbamazepine (Katwijk Farma B.V., Katwijk, The Netherlands) were kindly provided by Dr. B. Tuyl (Katwijk, The Netherlands). The anti-cancer drug etoposide (VP-16) and the pharmaceutical-grade barbiturates, butabarbital, hexobarbital and secobarbital were a gift from the Free University Academic Hospital (Amsterdam, The Netherlands). The phenylurea herbicides chlorobromuron, diuron, chlorotoluron, monolinuron and monuron were obtained as a gift from the Food Inspection Service (Amsterdam, The Netherlands). Serum was obtained from Nyegaard & Co (Oslo, Norway) and human plasma was obtained by collecting whole blood in heparinized tubes with subsequent centrifugation.

Demineralized water was purified in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system to obtain LC-grade water for use in eluents and standard solutions. Eluents were degassed in an ultrasonic bath under vacuum prior to use.

Procedures

Stock solutions of the model compounds were prepared by weighing and dissolving in methanol and stored at -20°C. The solutions were diluted with either water or blank serum, plasma, urine, river water or soft drink.

The reproducibility of the retention on the cartridges was studied for the model compound 3,5-dichlorophenol using aqueous 0.05% acetic acid as mobile phase, at a flow-rate of 1 ml min^{-1}. The influence of wetting of the cartridge by methanol prior to conditioning (with water or buffer, in order to remove the excess of methanol) and sampling was investigated by storing the cartridges in methanol or, alternatively, by on-line treatment with methanol via loop injection.

The contribution of the cartridge exchange module to extra-column band broadening

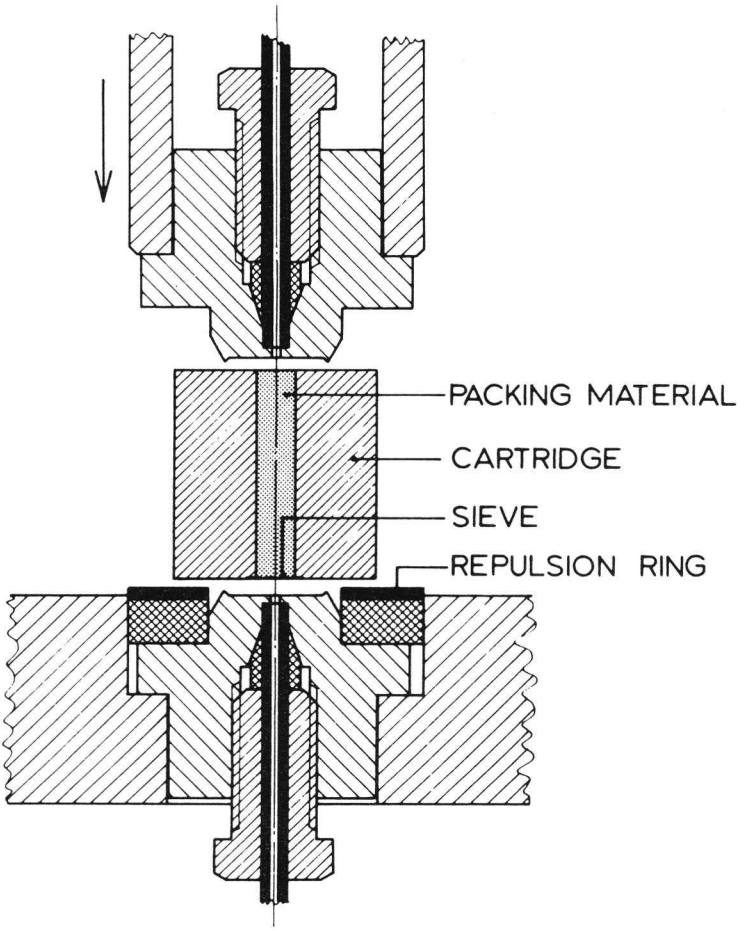

Fig. 1. Design of the pressure-resistant cartridge and the pneumatically controlled connection system.

was studied using the 20 cm analytical column and a mobile phase consisting of acetonitrile-0.11% acetic acid (55:45) at 0.4 ml min^{-1}. Under these conditions, 3,5-dichlorophenol showed a k' value of 3. The total system efficiency was evaluated by comparing a 10 µl direct loop injection of 1 mg ml^{-1} of 3,5-dichlorophenol, dissolved in the mobile phase with a preconcentration experiment of the model compound (1 ml of the same sample, after its 100-fold dilution with 0.05% acetic acid). The cartridges were wetted on-line via a 1 ml loop injection of methanol and conditioned with about 5 ml of 0.05% acetic acid prior to the actual preconcentration. Preconcentration of the analyte and purging with 5 ml of 0.05% acetic acid were done at 0.5 ml min^{-1}.

Design of the automated sample handling system

The heart of the automated sample handling system is the pressure-resistant cartridge which can be pneumatically mounted and connected to a high-pressure switching valve (Fig. 1). Because of the small inner diameter of the catridges, they are also compatible with narrow-bore (2-3 mm I.D.) LC systems. In addition, the length of the cartridge (10 mm) allows the introduction of highly protein-bound drugs, which will be released from the proteins with a recovery close to 100%, if an appropriate flow-rate is used[7]. The cartridges are placed in a transport strip, which is put into a reservoir (Fig. 2). The self-adjusting transport mechanism is shown in more detail in Fig. 3. The complete fully automated system shown in Fig. 4, further includes a purge pump, a solvent selection valve and an auto-sampler.

For actual operation, a cartridge is first wetted with methanol via the purge pump; then the solvent selection valve switches to water or a buffer solution in order to condition the catridge and to transfer the sample from the loop of the auto-sampler towards the cartridge. After sampling, the cartridge may be purged with the transfer solution or with another solution for clean-up. Next, the analytes are eluted from the cartridge in the forward-flush mode and the cartridge will be disposed of and replaced prior to the next sample. Because of the forward-flush elution, the cartridge also acts as a filter which prevents precipitates, proteins or other particulate matter to clog the connective outlet capillary.

The needle of the auto-sampler, and all capillaries through which the untreated sample is transferred, have inner diameters of at least 0.4 mm, to prevent clogging of the system.

The injection valve of the auto-sampler, the switching valve of the automated cartridge exchanger, the purge pump and the solvent selection valve are all controlled

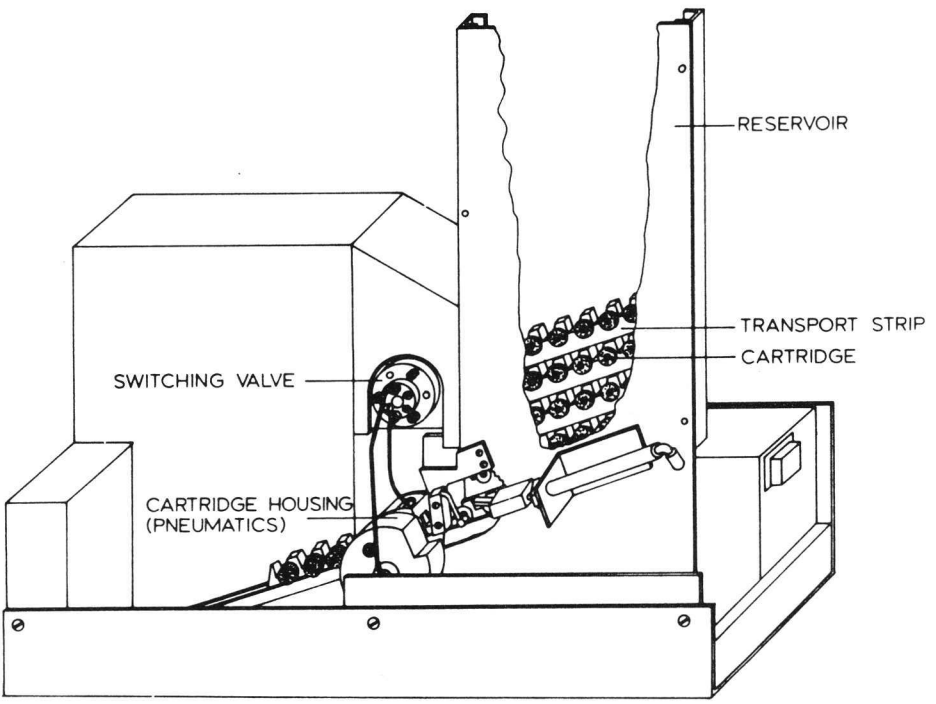

Fig. 2. Design of the automated cartridge exchange module.

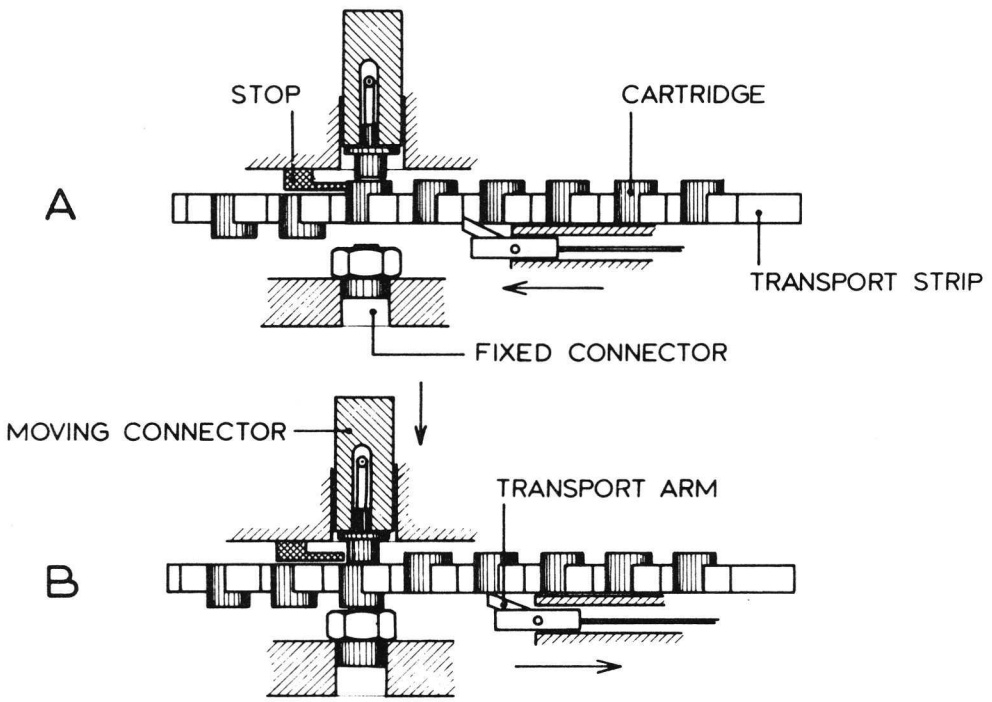

Fig. 3. Detail of the self-adjusting cartridge transport mechanism. (A) no connection, allowing cartridge transport; (B) closed, leak-tight connection with the switching valve.

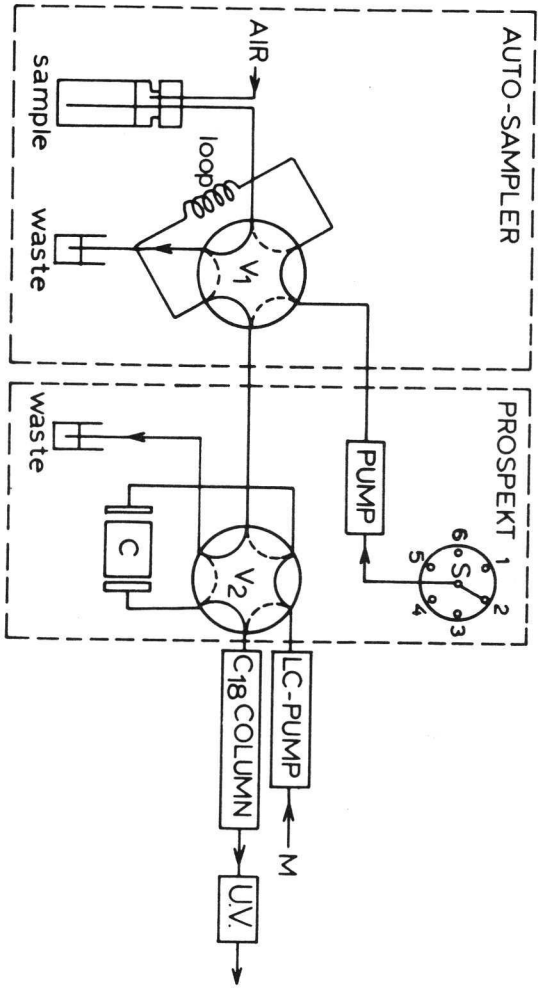

Fig. 4. Design of the automated sample handling (PROSPEKT) system. V_1, injection valve; V_2, high-pressure switching valve; S, six-port solvent selection valve; M, mobile phase; C, 10 x 2 mm I.D. cartridge, packed with 40 µm octyl-bonded silica.

via the auxillary program of the Promis auto-sampler. A flow-chart of the events of the system is given in the Appendix.

RESULTS AND DISCUSSION

Retention on the cartridges

The retention of 3,5-dichlorophenol on the catridges expressed in terms of its breakthrough volume, was found to be strongly dependent on the wetting of the sorbent prior to use (cf. Table II). In accordance with general findings wetting by methanol was an essential step in the solid phase extraction procedure. However, the volume of methanol used was found not to be very critical, which allowed on-line wetting via a loop injection by using, e.g., a vial filled with methanol in the satellite position of the Promis auto-sampler. However, we generally performed the on-line wetting via the purge pump and the solvent selection valve in our experiments. For our purpose the breakthrough volume and the rel. S.D. of the polar model compound are quite acceptable. One should note that in practical applications the total volume of sample and purge solvent is selected (far) below the actual breakthrough volume.

Table II. Breakthrough Volumes of 3,5-Dichlorophenol, According to the Procedure in the Experimental Section.

Conditions: 10 x 2 mm I.D. cartridges prepacked with 40 µm octyl-bonded silica; mobile phase, 0.05% acetic acid at 1 ml min^{-1}; injection volume, 33 µl (1.7 µg dichlorophenol); detection, UV at 220 nm.

Cartridge pretreatment	n	Breakthrough volume	
		V_B (ml)	rel. S.D. (%)
Not wetted	6	0.4	14
Stored in methanol	6	7.6	29
On-line wetted with 33 µl methanol	6	7.5	9

Contribution to extra-column band broadening

The influence of the cartridge system on the total system efficiency was investigated according to the procedure described in the experimental section. The results are shown in Table III. Obviously there is no significant decrease in the total system efficiency, when the PROSPEKT system is compared with a direct 10 µl loop injection. On the other hand, under comparable conditions the AASP showed relatively poor performance which resulted in a 30% loss of plate number and serious fronting of the 3,5-dichlorophenol peak. Possibly the relatively long connecting capillaries from the cartridge to the switching valve of the AASP and the larger length of the cartridges (2 cm) are responsible for this result.

Generally speaking, it is disadvantageous to use long precolumns when elution is carried out in the forward-flush mode[16], i.e., in the same direction as the sample application, but of course this mode is to be preferred in order to maintain the protective filter aspect of the disposable cartridges.

Table III. Influence of the Cartridge Exchange System on Total System Efficiency, According to the Procedures in the Experimental Section.

Method	No. of analyses	Plate number
10 µl loop injection	5	7900 ± 600
1 ml preconcentration, using the PROSPEKT system	6	7300 ± 600
1 ml preconcentration, using the AASP system**	6	5500 ± 400*

*Fronting

**Cartridges 20 x 2 mm I.D.

Filter action of the cartridge

The filter aspect was clearly demonstrated by the following experiment. 1 ml of whole blood, i.e., including red blood cells etc., was introduced to a cartridge in order to force a clogging of the system. This resulted indeed in a serious increase of back pressure which was attributed to the cartridge and not to clogging of valves, connective tubing, guard or separation column. The hypothesis of the cartridge being the only part responsible, was shown by exchanging the cartridge, which resulted in an immediate restoring of the original back pressure value.

Applications

In order to demonstrate the applicability range and analytical performance of the present system, unattended analyses of real samples were carried out for five different model systems, viz. anti-epileptic drugs in serum, an anti-cancer drug in plasma, barbiturates in urine, on-line trace enrichment of phenylurea herbicides from river water and the determination of caffeine in a soft drink.

Manual (off-line) sample preparation was restricted to the absolute minimum. For serum and plasma samples only buffer solution was added; the cola soft drink was degassed only and river water samples were filtered over a 0.8 μm membrane. With the urine samples, the most complex matrix under investigation, the elimination of interfering endogenous compounds caused some problems. In the final procedure we acidified urine with phosphoric acid to pH 3.5 and filtered the suspension thus obtained over a paper filter. Finally, the urine was diluted 1:1 with LC-grade water.

With most applications the samples were put into auto-sampler vials and the reservoir of the cartridge exchange module was filled with the disposable cartridges. However, in the on-line trace enrichment of the phenylureas, the samples were introduced via the solvent selection valve and the purge pump. The analytical conditions for the different applications are summarized in Table IV. Figures 5-9 show the chromatograms obtained with the system described above, for the different matrices under investigation. Each chromatogram shows (a) a direct loop injection; (b) an analysis of a standard solution via the automated sample handling system and (c) the analysis of a real sample automatically analyzed using the same conditions as in (b).

Table V summarizes the analytical data obtained with the PROSPEKT system. Data for the repeatability and the recovery are the mean values of 20 experiments; and for the memory effect the mean of 5 experiments. Recoveries were calculated by comparing the peak areas of the chromatograms (a) and (b), and (b) and (c), in order to be able to distinguish between the influence of the PROSPEKT system and the influence of the

Table IV. Conditions for the Applications using the Automated Sample Handling (PROSPEKT) System

Parameter	Anti-epileptic drugs	VP-16	Barbiturates
Matrix	serum	plasma	urine
Length analytical column (cm)	10+1	10+1	20+1
Eluent composition	MeOH-H_2O (45:55)	MeCN-H_2O (25:75)	MeCN-H_3PO_4/pH 2.7 (3:7)
Flow-rate (ml min^{-1})	0.8	0.5	0.5
UV detection	195	230	254
Sample composition	1 ml stock of drugs + 1 ml 2M phosphate (pH 3.5) + 18 ml blank serum	1 ml stock of VP-16 + 1 ml 2M phosphate (pH 3.5) + 2 ml blank plasma	1 ml stock of drugs + 19 ml filtered urine (pH 3.5) + 20 ml water
Sample volume (µl)	20	100	20
Concentration (µl ml^{-1})	5	0.2	5
	(a) MeOH; (b) H_3PO_4 (pH 2); (e) eluent	(a) MeOH; (b) H_3PO_4 (pH 2); (e) eluent	(a) MeOH; (b) H_2O; (c) 10% MeCN; (e) eluent
Flow-rate pure pump (ml min^{-1})	1	1	1
Timed events (solvent, ml) - wetting - conditioning - sampling - purging - desorption reset	 (a) 0.5 (b) 2 loop (b) 1.2 (e) 0.55	 (a) 0.5 (b) 2 loop (b) 1.2 (e) 0.33	 (a) 0.5 (b) 2 loop (b) 5.5; (c) 0.5 (e) 1.0

Herbicides	Caffeine
river water	cola
20+1	10+1
MeOH-0.02 M phosphate buffer/pH 7 (45:55)	MeCN-H_2O (15:85)
0.4	0.5
245	280
1 ml stock of herbicides 99 ml river water	degassed cola
10,000	20
0.01	blank 60; spike 120
(a) MeOH; (b) H_2O; (c) Sample; (d) H_2O; (e) eluent 1	(a) MeOH; (b) H_2O; (e) eluent 1
(a) 0.5 (b) 1 (c) 10 (d) 2 (e) 2.4	(a) 0.5 (b) 1 loop (b) 1 (e) 0.5

Table V. Analytical Data for the Applications with the Automated Sample Handling (PROSPEKT) System.

Conditions are given in Table IV, the system used is further described in the experimental section; data based on peak area measurements; memory effect specified for the auto-sampler, ≤ 0.5 %.

		Recovery (%)		
Analytes	Repeatability (% RSD)	Stand. vs. loop	Sample vs. standard	Memory (%)
n =	20	20	20	5
Primidone	2.5	112	89	≤ 0.5
Phenobarbital	4.6	104	89	≤ 0.5
Phenytoin	2.9	98	98	≤ 0.5
Carbamazepine	4.3	103	101	1.0
VP-16	5.1	92	61	≤ 0.5
Butabarbital	4.2	99	98	≤ 0.5
Hexobarbital	5.1	96	86	≤ 0.5
Secobarbital	3.5	104	115	≤ 0.5
Monuron	2.4	89	95	≤ 0.5
Monolinuron	1.5	89	104	≤ 1.0
Chlorotoluron	3.1	97	94	≤ 0.5
Diuron	3.2	96	95	≤ 0.5
Chlorobromuron	4.8	96	97	≤ 0.5
Caffeine	3.2	87	97	≤ 0.5

matrix, on the final recovery. The memory effect was studied by the injection of a blank solution directly after the analysis of a real sample, and performing the automated analysis at a 10-fold increased sensitivity of the UV-absorbance detector. In general, the analytical data for the 14 analytes in five different matrices as shown in Table V are quite satisfactory with a repeatability ranging from 1.5 to 5.1% RSD (n = 20); recoveries (n = 20) ranging from 87 to 115% (except for VP-16; cf. below) and a memory effect (n = 5) within the specification of the auto-sampler itself, i.e., £ 0.5% (except for two compounds).

As regards the anti-epileptic drugs (cf. Fig. 5) the strong protein binding, usually observed for phenytoin and carbamazepine, did not influence the recovery at all. However, the samples and the purge solvent had to be acidified in order to obtain a good recovery for phenobarbital. This phenomenon, as well as, the recoveries for the other anti-epileptic drugs, is in excellent agreement with the results obtained by other authors[10,12]. To control the reliability of the system for the determination of these drugs in serum, a series of 96 samples was run unattended overnight. Chromatograms were evaluated by computer and the repeatability was calculated from the individual peak height data. The repeatability was better than 3.4-5.2% RSD for the anti-epileptic drugs studied. No blocking of the guard or analytical column occurred and the memory effect was less than 0.5% for all but one of these drugs (1%).

The recovery of VP-16 in plasma (cf. Table V and Fig. 6) was found to be relatively poor (61%), but the repeatability was still acceptable, for an unattended analysis of real samples. This relatively low recovery was only observed for real samples which is in good agreement with earlier findings for a 5 x 1.1 mm I.D. precolumn packed with the same material[17].

The on-line trace enrichment of phenylurea herbicides from river water samples (Fig. 7) and the determination of caffeine in cola (Fig. 8) showed a good overall performance with repeatability from 1.5-4.8% RSD, recoveries ranging from 89-104% and a memory effect less than 0.5% for all but one of the herbicides (1%).

The determination of barbiturates in urine (Fig. 9) was found to be more critical. Although the analytical data are still acceptable (cf. Table V) at this realistic concentration level, it is obvious that when a lower detection limit is desired, the selectivity will have to be improved. This can be achieved by using a more selective detection mode or via the on-line dual precolumn approach described by De Jong et al.[18]. These authors used a precolumn packed with a hydrophobic resin in series with an ion-exchange precolumn in order to selectively eliminate organic and inorganic interferences, thus allowing the LC determination of barbiturates in urine with (non-selective) UV absorbance detection.

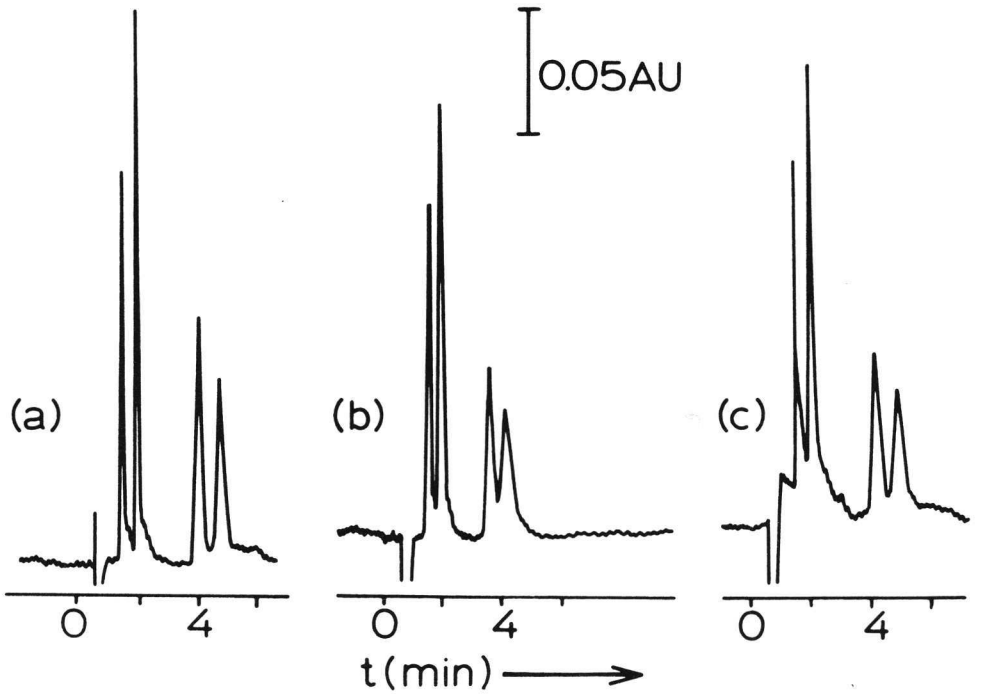

Fig. 5. Chromatograms of the anti-epileptic drugs primidone, phenobarbital, phenytoin and carbamazepine. (a) direct loop injection; (b) as (a), but using the system described in Fig. 4; and (c) spiked serum sample, analyzed as (b). Conditions, see Table IV.

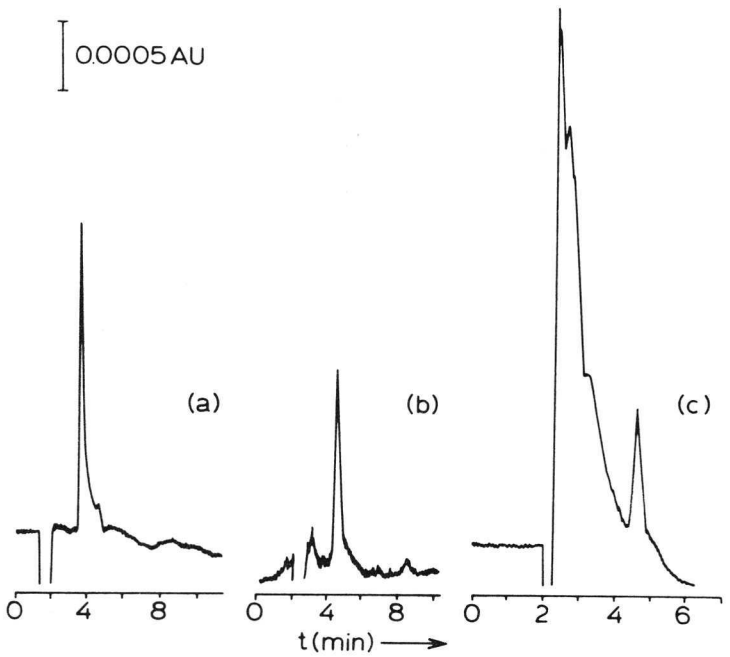

Fig. 6. Chromatograms of the anti-cancer drug VP-16. (a) direct loop injection; (b) as (a), but using the system described in Fig. 4; and (c) spiked plasma sample, analyzed as (b). Conditions, see Table IV.

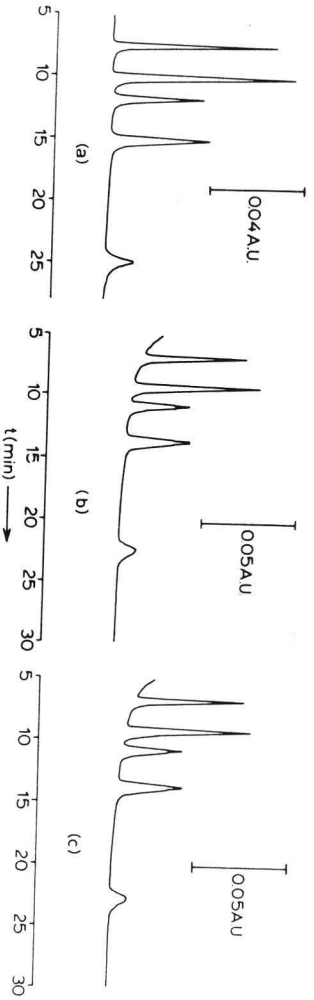

Fig. 7. Chromatograms of the phenylurea herbicides monuron, monolinuron, chlorotoluron, diuron and chlorobromuron. (a) direct loop injection; (b) 10 ml on-line trace enrichment using the system described in Fig. 4; and (c) spiked river water sample, analyzed as (b).
Conditions, see Table IV.

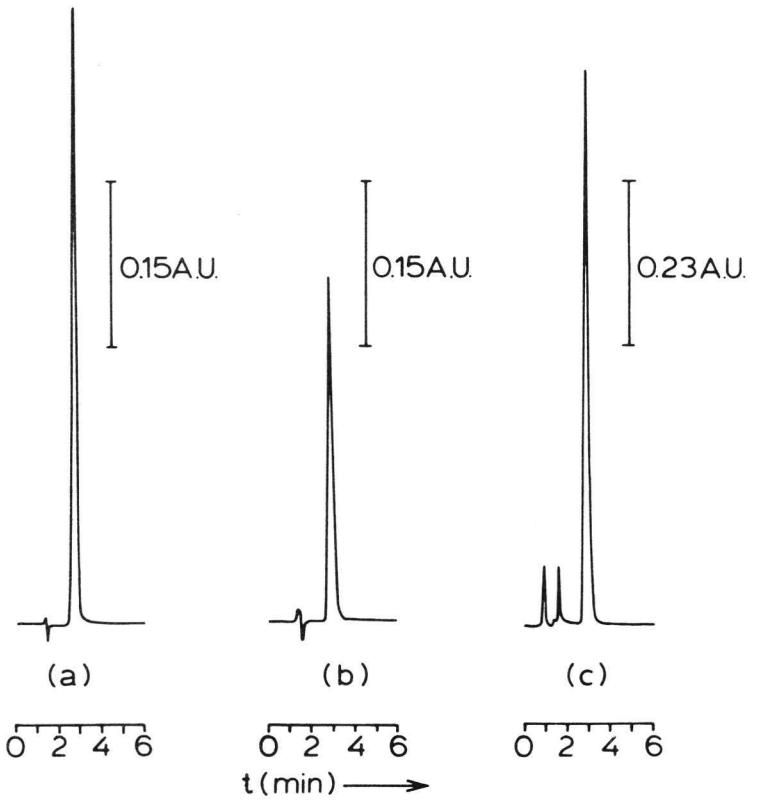

Fig. 8. Chromatograms of caffeine. (a) direct loop injection; (b) cola, analyzed using the system described in Fig. 4; (c) spiked cola, analyzed as (b). Conditions, see Table IV.

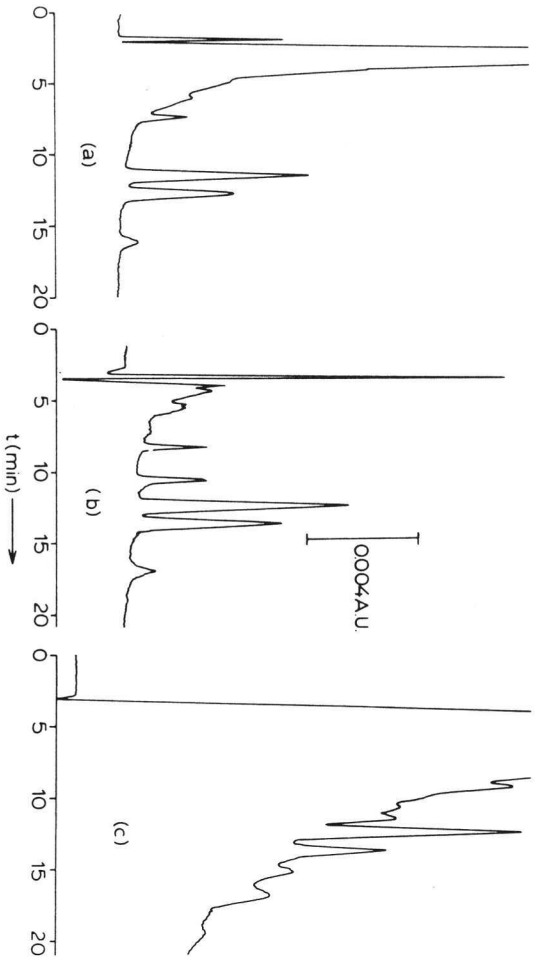

Fig. 9. Chromatograms of the barbiturates butabarbital, hexabarbital and secobarbital. (a) direct loop injection; (b) as (a), but using the system described in Fig. 4; and (c) spiked urine sample, analyzed as (b).
Conditions, see Table IV.

CONCLUSIONS

A fully automated sample handling system has been developed for liquid chromatography which combines the advantages of a disposable cartridge system with its inherent constant quality assurance, and precolumn technology with its high automation potential. The present design represents a prototype of a new generation of automated sample handling systems where the samples, in principle, only have to be applied to the instrument.

In order to achieve this end, a new low-cost pressure-resistant cartridge packed with 40 μm octyl-bonded silica was developed; this packing shows acceptable reproducibility as was demonstrated by the retention for a polar model compound. Extra-column band broadening caused by this system was found to be negligible when combined with a 20 cm analytical column.

Five different sample matrices were investigated to demonstrate the potential of the present system for unattended routine analyses. Repeatability and recovery were found to be satisfactory for the determination of drugs in serum, plasma and urine, for the trace-level determination of herbicides in river water and for the determination of caffeine in cola. In addition the memory effect was found to be the same as the specification for the auto-sampler used (£ 0.5%) for all but two compounds (1%).

It is obvious that the potential of an automated sample handling system based on solid phase extraction can only be fully exploited if a wide range of cartridges packed with selective sorbents becomes commercially available. In addition, it is the intention to provide interesting additional options such as switching valves, allowing the use of a dual-precolumn approach[18], e.g., the combination of a non-selective reversed phase-type precolumn with a selective ion-exchange cartridge; solvent selection valves for the on-line trace enrichment from large sample volumes; and a low-cost sampler, instead of the sophisticated auto-sampler used in this study.

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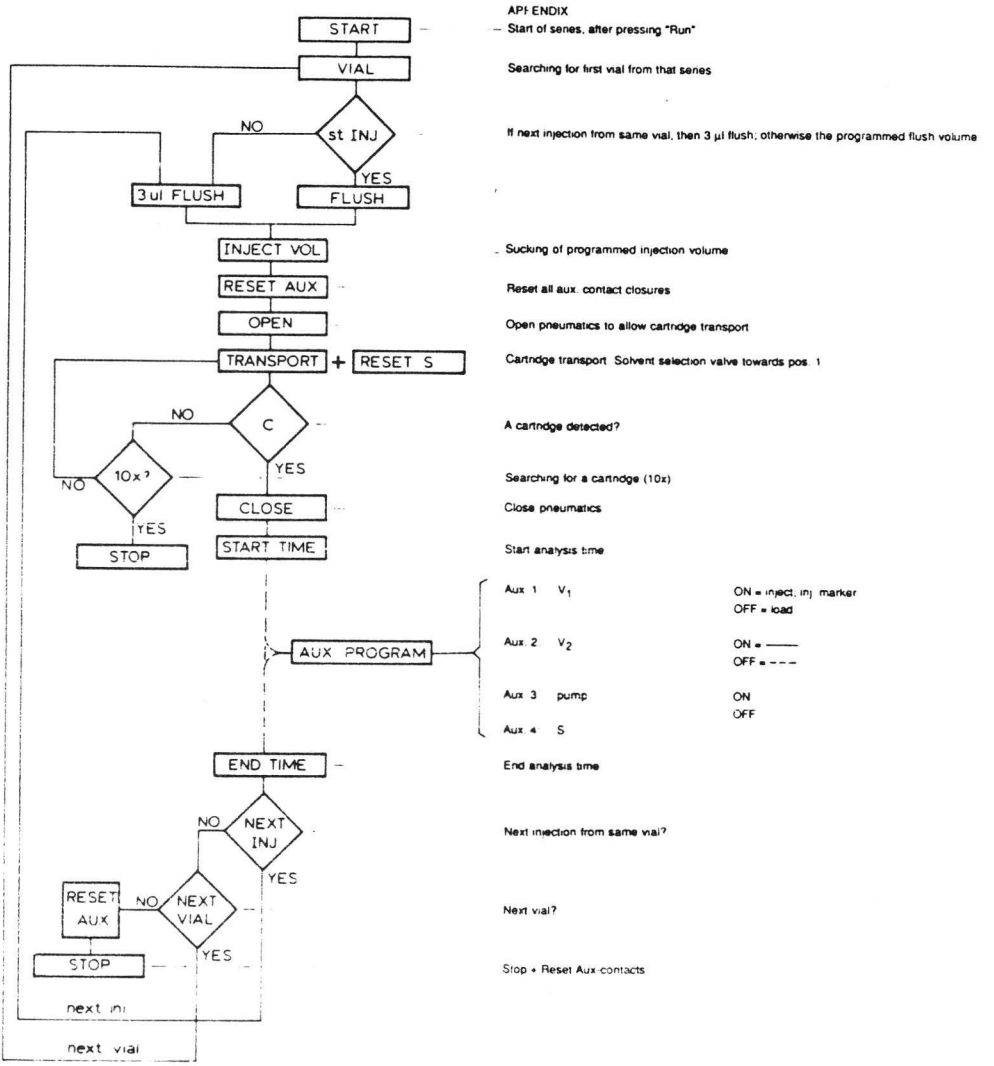
References

1. R.W. Frei, M.W.F. Nielen and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 25 (1986) 3.
2. R.D. McDowall, J.C. Pearce and G.S. Murkitt, J. Pharm. Biomed. Anal., 4 (1986) 3.
3. Baker-10 SPE' Application Guide, Vol. I and II, Phillipsburg, VA, U.S.A.
4. Sorbent Extraction Technology Handbook, K.C. van Horne (ed.), Analytichem International, Harbor City, CA, U.S.A.
5. R.C. Williams, Int. J. Environ. Anal. Chem., 18 (1984) 37.
6. J.C. Pearce, J.A. Kelly, K.A. Fernandes, W.J. Leavens and R.D. McDowall, J. Chromatogr., 353 (1986) 371.
7. K. Zech and R. Huber, J. Chromatogr., 353 (1986) 351.
8. P.J. Arnold and O.V. Stetten, J. Chromatogr., 353 (1986) 193.
9. U. Juergens, J. Chromatogr., 310 (1984) 97.
10. A. Nazareth, L. Jaramillo, B.L. Karger, R.W. Giese and L.R. Snyder, J. Chromatogr., 309 (1984) 357.
11. W. Roth, J. Chromatogr., 278 (1983) 347.
12. U. Juergens, Int. J. Environ. Anal. Chem., 25 (1986) 221.
13. M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 317 (1984) 557.
14. M.W.F. Nielen, J. de Jong, R.W. Frei and U.A.Th. Brinkman, Int. J. Environ. Anal. Chem., 25 (1986) 37.
15. M.W.F. Nielen, H.E. van Ingen, A.J. Valk, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., submitted for publication (1986).
16. C.E. Goewie, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 301 (1984) 325.
17. M.W.F. Nielen, E. Sol, R.W. Frei and U.A.Th. Brinkman, J. Liq. Chromatogr., 8 (1985) 1053.
18 J. de Jong, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 381 (1986) 431.

APPENDIX


START
VIAL
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YES
STOP
CLOSE
START TIME
AUX PROGRAM
END TIME
NO
NEXT INJ
YES
RESET AUX
NO
NEXT VIAL
YES
STOP
next inj
next vial
APPENDIX
Start of series, after pressing "Run"
Searching for first vial from that series
If next injection from same vial, then 3 µl flush; otherwise the programmed flush volume
Sucking of programmed injection volume
Reset all aux. contact closures
Open pneumatics to allow cartridge transport
Cartridge transport. Solvent selection valve towards pos. 1
A cartridge detected?
Searching for a cartridge (10x)
Close pneumatics
Start analysis time
Aux 1 V1
ON = inject, inj. marker
OFF = load
Aux 2 V2
ON = ——
OFF = - - -
Aux 3 pump
ON
OFF
Aux 4 S
End analysis time
Next injection from same vial?
Next vial?
Stop + Reset Aux-contacts

APPENDIX B

Dual-precolumn automated sample handling using the PROSPEKT system.

Introduction. As was outlined above, there are several reasons to combine the advantages of precolumn/cartrdige exchange and the automation aspects of precolumn technology.

However, for more selectivity at the sample handling side, the use of more than one precolumn is sometimes unavoidable. Usually a non-selective reversed-phase type precolumn is combined with a second, more selective precolumn packed with, e.g., ion-exchangers[1,2] or metal-loaded phases[3]. The regeneration of these selective sorbents is often tedious, time-consuming and sometimes even impossible. Consequently, this cartridge should be automatically exchanged.

On the other hand, if off-line manipulations are restricted to the absolute minimum, then the first, non-selective, precolumn should be also exchanged frequently, because of its important filter function (cf. above). So the question is which of the two cartridge-types will be exchanged.

Of course this dilemma can be easily solved by coupling two PROSPEKT systems in series, thereby allowing the exchange of both cartridges. A more economical approach is described in Fig. A1, using the two optional high-pressure switching valves of the PROSPEKT.

Procedure. The samples are placed in the auto-sampler and the cartridge reservoir is filled with the transport strip(s) containing the non-selective and selective cartridges in the order ABABAB.....

The first cartridge is wetted with methanol via the purge pump, then the solvent selection valve switches to water or a buffer solution in order to condition the cartridge and to transfer the sample from the loop of the auto-sampler towards the cartridge. After sampling, the cartridge may be purged with the transfer solution or with another solution for clean-up. Next, the solvent selection valve switches to a suitable eluent and simultaneously valve V_4 (cf. Fig. A1) is switched. In this step, the compounds of interest are eluted from the cartridge and stored in a loop, mounted at valve V_3, by carefully controlling the position of valve V_4.

Next, the cartridge can be exchanged and replaced by the second, more selective, cartridge. Again the solvent selection valve switches to methanol and/or aqueous buffer solutions in order to wet and to condition the cartridge prior to use. Then valve V_3 (cf. Fig. A1) is switched and the eluate from the first cartridge which was stored in the loop at V_3, is loaded on the second cartridge. After sample loading, the second cartridge may also be purged for further clean-up. Finally, valve V_2 is switched and the purified sample

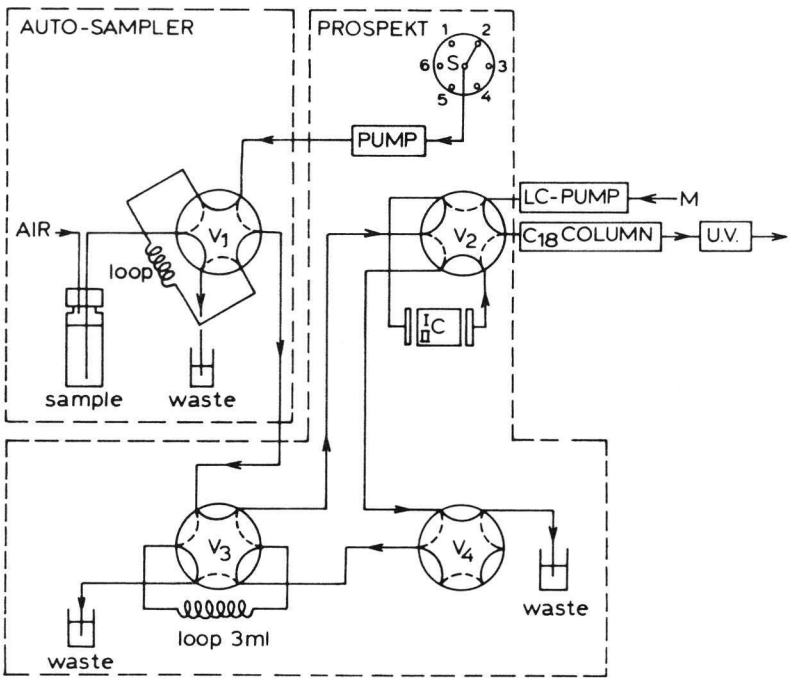

Fig. A1. Design of the dual-precolumn sample handling system. V_1, injection valve; V_2, V_3 and V_4, high-pressure switching valve; S, six-port solvent selection valve; M, mobile phase; ^{I}C and ^{II}C, 10 x 2 mm I.D. cartridges packed with a non-selective and a selective sorbent, respectively.

is eluted by the mobile phase towards the analytical column.

References

1. *This thesis,* Chapter 2.
2. *This thesis,* Chapter 3.
3. *This thesis,* Chapter 4.

Chapter 7. Conclusions and further developments

The use of precolumn technology can provide us with fully automated sample handling and trace-enrichment procedures for aqueous environmental and biomedical samples. A series of sorbents for either off-line or on-line solid phase extractions has been commercialized and an increasing amount of equipment is becoming commercially available. Concerning on-line precolumn design, prepacked cartridges seem to be preferred by manufacturers and non-university customers, despite the advantages of manual packing of a wide choice of sorbents. It is to be expected that a major part of the growing amount of off-line solid phase extraction applications will be converted to on-line systems in the future, because of the automation aspect.

One of the serious disadvantages of sample handling and trace enrichment on the polystyrene divinylbenzene materials such as XAD-2 and PRP_1, and the C_8 and C_{18} chemically bonded phases is the inherent lack of selectivity. In such cases, one interesting approach is the application of more selective detection modes[1] or column switching[2]; however, selectivity can also be increased by using selective sorbents such as ion-exchangers[3] and metal-loaded phases[4]. Several research groups and manufacturers are developing more selective sorbents for solid phase extraction, for example phenylboronic acid-bonded silica[5] for the selective isolation of catecholamines from urine.

Apart from the use of immobilized enzymes for the on-line hydrolysis of conjugates[6] from urine, there might be also some potential for the specific isolation of particular (classes of) compounds. However, one should always keep in mind the existence of immuno-assays which will have a superior potential in many cases.

On-column derivatization using solid phase extraction columns is expected to be applied more often. The sorbent may be impregnated with a reagent[7], prior to loading of the sample. As an alternative, a sample can be firstly immobilized on a suitable sorbent and then flushed with the derivatization reagent. Finally after a certain reaction time, the excess of the reagent can be removed and the derivatives eluted on-line to the LC separation column.

The on-line fractionation of complex mixtures by using a series of precolumns packed with different types of sorbents[9], will certainly gain interest. Coupling precolumns packed with normal-phase and reversed-phase type materials[10] will be very interesting for the analysis of solid samples such as soil and foodstuffs. With such samples, extracts dissolved in organic solvents are often obtained which are, therefore,

not directly compatible with reversed phase precolumns and separation systems. Precolumn technology coupled with vacuum or gas-flushing systems to remove undesirable solvents seems to be a valuable tool to realize the normal phase-reversed phase coupling and also to facilitate the interfacing between automated sample handling via on-line LC precolumn technology and separation by means of capillary gas chromatography[11].

Another field to be explored is the coupling of conventional-size precolumn systems with a final micro-precolumn prior to a miniaturized LC separation column. An interesting, and rather obvious, example is the use of a non-selective sorbent in a relatively large precolumn with a highly selective -- and much more expensive -- sorbent in the micro-precolumn[12].

Finally, it is to be expected that field-sampling on small precolumn-cartridges will become more popular. Close cooperation between analytical chemists and users is an important condition to realize that goal. Conceivably, precolumn-cartridges will be integrated in syringe systems for on-site environmental sample loading and storage, and for taking blood samples in physicians offices or hospitals and isolating compounds of interest simultaneously. Samples can easily be stored on the precolumns and finally sent to a central laboratory where they are put into an automated precolumn-cartridge processor[13] and analyzed.

References

1. R.W. Frei, H. Jansen and U.A.Th. Brinkman, Anal. Chem., 57 (1985) 1529A.
2. K. Ramsteiner, Int. J. Environ. Anal. Chem., 25 (1986) 49.
3. *This thesis*, Chapters 2 and 3.
4. *This thesis*, Chapter 4.
5. Bond-elut®, Analytichem, Harbor City, CA, U.S.A.
6. L.D. Bowers and P.R. Johnson, Anal. Biochem., 116 (1981) 111.
7. M. Rosenfeld, M. Mareika-Russell and A. Phatak, J. Chromatogr., 283 (1984) 127.
8. A.H.M.T. Scholten, B.J. de Vos, J.F. Lawrence, U.A.Th. Brinkmand and R.W. Frei, Anal. Lett., 13 (1980) 1235.
9. *This thesis*, section 2.2.
10. A.T. Boo and J. Krohn, J. Chromatogr., 301 (1984) 335.
11. E. Noroozian, F.A. Maris, M.W.F. Nielen, R.W. Frei, G.J. de Jong and U.A.Th. Brinkman, presented at 16th Int. Symp. on Chromatography, Paris (1986).
12. K. Slais, M.W.F. Nielen, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr.

(1986) submitted for publication.

13. *This thesis*, Chapter 6.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling van automatische monstervoorbehandelingstechnieken in de vloeistofchromatografie. Speciale aandacht is gewijd aan de selectiviteit van deze technieken — een essentieel punt in de sporenanalyse van organische stoffen in milieumonsters en lichaamsvloeistoffen.

In dit werk is gebruik gemaakt van vloeistof-vast extracties op verscheidene sorbentia, gepakt in kleine voorkolommen. In Hoofdstuk 1 komen de basisprincipes van deze techniek alsmede het gebruik in automatische systemen (voorkolomtechnologie) aan de orde.

Hoofdstuk 2 beschrijft het gebruik van kationenwisselaars voor selectieve monstervoorbehandeling en preconcentratie van polaire anilines vanuit rivierwater. Storende anorganische kationen worden vóór de analyse verwijderd met behulp van neerslag- en complexvormingsreacties (2.1). Hetzelfde systeem, uitgebreid met een tweetal voorkolommen gevuld met hydrofobe pakkingsmaterialen, werd toegepast voor de analyse van industrieel afvalwater waarbij simultaan een fractionering en preconcentratie plaatsvindt. Een interessant detail daarbij is het gebruik van een diode-array detector voor de identificatie van microverontreinigingen in deze complexe monsters (2.2).

In Hoofdstuk 3 komen anionenwisselaars aan de orde die worden toegepast voor de bepaling van sporen fenol in rivierwater (3.1) en de clean-up van barbituraten vanuit urine (3.2). Terwijl de gezochte verbindingen worden gevangen op een voorkolom gepakt met een hydrofoob sorbens, worden storende anorganische anionen on-line verwijderd. Daarna kunnen de verbindingen zonder bezwaar worden geherconcentreerd op een voorkolom gepakt met een anionenwisselaar.

Hoofdstuk 4 behandelt het gebruik van met metaal beladen voorkolommen voor selectieve monstervoorbehandeling. Eerst wordt de principiële toepasbaarheid gedemonstreerd via een relatief eenvoudig voorbeeld, de preconcentratie van een thiol op een kwik(II) fase (4.1). Vervolgens (4.2) wordt een drietal verschillende commercieel beschikbare sorbentia geëvalueerd voor monstervoorbehandelingsmethoden gebaseerd op ligandenwisselings chromatografie. De toepasbaarheid in de milieuanalyse wordt gedemonstreerd door de selectieve preconcentratie van het fenylureum herbicide buturon op een met zilver(I) beladen fase. Tenslotte wordt de waarde van dit type voorkolom voor de selectieve bepaling van verbindingen in lichaamsvloeistoffen gedemonstreerd met de analyse van ethynylhormonen in urine. Storende bestanddelen worden verwijderd door de hormonen eerst te vangen op een hydrofobe voorkolom, gevolgd door een selectieve herconcentrering op een met zilver(I) beladen fase (4.3).

In hoofdstuk 5 wordt aandacht geschonken aan de miniaturisering van voorkolommen. Met name het ontwerp van de voorkolom blijkt een kritische factor, zeker als de analyse van onbehandelde plasmamonsters als randvoorwaarde wordt gesteld. In de beschreven toepassing wordt de selectiviteit niet verkregen door gebruik te maken van een selectief sorbens, maar door de toepassing van een (relatief) groot spoelvolume.

Hoofdstuk 6 beschrijft een recente ontwikkeling, de integratie van voorkolomtechnologie met automatische cartridge wisseling. Het is immers niet altijd gewenst of mogelijk om een voorkolom na regeneratie opnieuw te gebruiken. De beschreven ontwikkeling heeft geleid tot een commercieel verkrijgbaar instrument voor de automatische analyse van onbehandelde plasma-, serum- en urinemonsters.

Tenslotte worden in Hoofdstuk 7 enige conclusies getrokken en mogelijk toekomstige ontwikkelingen aangegeven.

Dankwoord

Zonder de inspanningen van de volgende mensen was het nooit wat geworden met dit proefschrift:

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Spark Holland B.V. heeft de omslag van dit proefschrift ontworpen en ter beschikking gesteld.

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